PERIPHERAL BLOOD T CELL GENE EXPRESSION PROFILES IN THE EARLY POST RENAL TRANSPLANT PERIOD

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SIGNED DECLARATION

Thesis: "Peripheral Blood T Cell Gene Expression Profiles In The Early Post Renal Transplant Period."

I hereby declare and affirm that the above-mentioned thesis is entirely my own work and composition, and that I have not submitted this thesis in candidature for any other degree, diploma or professional qualification.

Date: 31st August 1999

LIST OF ABBREVIATIONS

APC	antigen presenting cell
cDNA	complementary deoxyribonucleic acid
CTL	cytotoxic T lymphocyte
CD	cluster of differentiation
DEPC	diethyl pyrocarbonate
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescein-activated cell scan
FasL	fas ligand
FCS	fetal calf serum
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-macrophage colony stimulating factor
GrB	granzyme B
HLA	human leucocyte antigen
IFN	interferon
ICAM	intercellular adhesion molecule
Ig	immunoglobulin
IL	interleukin
l	litres
LAK	lymphokine-activated killer
MAF	macrophage activating factor
MHC	major histocompatibility complex
µl	microlitres
ml	millilitres
mRNA	messenger ribonucleic acid
NK	natural killer
PCR	polymerase chain reaction
PBS	phosphate buffered saline
ra	receptor antagonist
rpm	revolutions per minute
RT	reverse transcription/reverse transcriptase
Taq	Thermus aquaticus
TCR	T cell receptor
Th	T helper
TNF	tumour necrosis factor
VCAM	vascular cell adhesion molecule

ABSTRACT

Acute rejection remains an important cause of early renal allograft loss and a risk factor for long term graft survival despite the substantial improvement in graft survival over the past 3 decades. Immunological monitoring of alloreactivity following organ transplantation has been the longstanding aim of transplant clinicians. Most studies monitoring cytokine gene expression were single time point "snap-shots" and hence conflicting findings abound in the literature with no consensus about which cytokines are markers or mediators of acute rejection.

Sequential monitoring of peripheral T lymphocyte gene expression was performed in renal transplant recipients in the first 6 weeks following transplantation. The level of gene expression was correlated with clinical events, with emphasis on changes occurring during acute rejection. Forty-three renal transplant patients were monitored. Twenty-eight did not reject while 15 did. Peripheral T cells were isolated at fixed time points within the first 6 weeks following transplantation, with additional samples taken at the time of acute rejection and following anti-rejection therapy. A robustly tested RT-PCR technique combined with a highly sensitive ELISA method of detecting digoxigenin-labelled PCR products was used to semi-quantitatively detect the sequential changes in interleukins (IL) 2, 4, 5, 10, 13, interferon (IFN)-γ, and the gene expression of 2 cytotoxic T lymphocyte (CTL) activation markers, granzyme B (GrB) and fas ligand (fasL).

Overall, T helper (Th)-2 cytokines (IL-4, IL-5, IL-10 and IL-13) showed the most interesting and varied changes in gene expression profiles following transplantation, while Th-1 cytokines (IL-2 and IFN-γ) showed unexpected changes and the CTL activation markers (GrB and FasL) showed the most random changes.

The most significant finding was that peripheral T cell IL-4, IL-5 and IL-13 gene expression increased at the time of acute rejection and decreased following successful anti-rejection therapy. While clear differences in the gene expression

profiles between non-rejectors and rejectors were seen for IL-5 and IL-13, there were more similarities in the profiles between the two groups for IL-4. The increase in IL-5 and IL-13 expression in the rejectors prior to, and during acute rejection, and the subsequent return of both cytokines to their respective patterns for the non-rejectors, suggest that these 2 cytokines may be important markers of acute rejection. The similar decrease in IL-4 gene expression below its pre-transplant baseline in the first week following transplantation in both non-rejectors and rejectors before returning to the baseline, and a similar reduction in expression below the baseline following anti-rejection therapy before returning again to the pre-transplant level suggest that IL-4 was more likely a sensitive marker of the changing level of immunosuppression rather than of the changes in alloreactivity. IL-4, IL-5 and IL-13 also had different gene expression profiles in the non-rejectors. Both IL-5 and IL-13 had a flat post-transplant gene expression profile, with IL-5 consistently below its pre-transplant baseline at all post-transplant time points while IL-13 remained at its baseline level throughout. IL-4 on the other hand had a variable profile, being below its baseline during the first week post-transplant before returning to its baseline subsequently.

In contrast, IL-10 gene expression profile was totally opposite to that for IL-4, IL-5 and IL-13 in both non-rejectors and rejectors. In the non-rejectors, IL-10 gene expression was consistently above its pre-transplant baseline at all post-transplant time points. At the time of acute rejection and even by 2 weeks post-transplant, IL-10 gene expression had decreased relatively by returning to its pre-transplant baseline, and with successful anti-rejection therapy, IL-10 expression increased above its baseline. The consistently high level of IL-10 expression in non-rejectors and the relative fall in IL-10 prior to, and during acute rejection, suggest that a high IL-10 level may be important for graft acceptance.

As for the Th-1 cytokines (IL-2 and IFN-γ), both showed similar flat profiles in non-rejectors with their gene expression levels remaining at their respective pre-transplant baselines at all post-transplant time points. In the rejectors, both cytokines showed significant and paradoxical reduction in the level of their

expression during the first week following transplantation and immediately following anti-rejection therapy, but returned to their pre-transplant baselines at all other time points. The unexpected fall in IL-2 and IFN-γ gene expression at several post-transplant time points in the rejectors only may represent a preferential sequestration of these Th-1 cells within the renal allograft prior to acute rejection. The 2 CTL activation markers (GrB and FasL) showed variable levels of gene expression in both non-rejectors and rejectors, with no apparent correlation with the clinical course following transplantation.

In conclusion, changes in some peripheral T cell cytokine gene expression correlated closely with clinical events. The use of sequential monitoring of peripheral T cell cytokine gene expression has the potential to provide a deeper understanding of the complex interactions of cytokines following transplantation, during acute rejection and the response to anti-rejection therapy.

Chapter 1: INTRODUCTION

This chapter sets out the basis for the research project on which this thesis is based and discusses the various contribution from the extensive but often conflicting literature on the role of cytokines and cytotoxic T lymphocyte activation markers on acute allograft rejection. In addition, the rationale for the development of a technically robust but relatively simple, reliable and sensitive semi-quantitative polymerase chain reaction-based method of monitoring changes in the levels of cytokine and cytotoxic T lymphocyte activation marker gene expression in peripheral blood T cells is outlined. In this study, this methodology was developed and applied as a means of employing peripheral blood in monitoring the changes in alloreactivity following adult clinical renal transplantation.

1.1 Background

Since the pioneering days over 30 years ago, kidney transplantation has progressed rapidly from what was an essentially unsuccessful experimental procedure to the successful and routine surgical procedure it is today, transforming the lives of an estimated 35,000 patients with chronic renal failure worldwide each year [Allen and Chapman, 1994].

Short and long term kidney graft survival rates have progressively improved over the past decade [Cecka, 1997]. However, up to 15% of grafts are lost within the first year and the persistent annual attrition rate of approximately 5% leads to unsatisfactory long-term survival with up to 60% of first cadaveric renal allografts failing 5 years following transplantation, and there is a further increased risk of failure with subsequent grafts [Morris, 1994b; Cecka, 1997].

While graft failures are not always the consequence of immunological insults, it is well established that the occurrence of early rejection episodes is a strong predictor of late kidney failure [Cuturi et al, 1994]. In terms of graft survival rates, the most recent UNOS Scientific Renal Transplant Registry's ten-year results of kidney transplantation [Cecka, 1997] has shown that transplant recipients who were rejection-free at discharge from the hospital had a statistically significant 13% higher one-year graft survival rate compared with those who experienced one or more rejection episodes during their transplant hospitalization. Acute rejection is also considered to be the single most important risk factor for the development of chronic rejection, the commonest cause of long-term allograft failure [Almond et al, 1993]. Although the estimated half-life of kidney grafts (time taken for 50% of functioning grafts at 1 year to fail) has improved in the past 10 years, the difference between patients experiencing early rejection and those without has nevertheless increased by over 70% (0.7 to 1.2 years) as demonstrated by the most recent estimated half-life of grafts (1994-1996) for patients without rejection being 10.3 years, and 9.1 years for those with one or more rejection episodes, compared with 8.3 years and 7.6 years respectively for 1988-1990 [Cecka, 1997].

Clinical immunosuppression, likewise, has undergone a gradual parallel transformation, both in terms of an increasing spectrum of immunosuppressive agents available for clinical use, as well as a better understanding in the mode of action of these agents [reviewed in Wilkinson et al, 1994]. Since the introduction of cyclosporin A into renal transplantation by Calne and colleagues in Cambridge in 1978, its major beneficial impact on the improved graft survival in clinical transplantation throughout the 1980s has led to its being the cornerstone of modern immunosuppression [reviewed in Morris, 1994a].

In recent years, the emphasis in the development of immunosuppressive agents has shifted from the older, established drugs like steroids and azathioprine which are essentially non-specific in their mode of action, and even the relatively more specific cyclosporin A, to the newer, more powerful agents like mycophenolate mofetil (which targets specific metabolic pathways of lymphocytes by inhibiting the enzyme

inosine monophosphate dehydrogenase required for purine synthesis) and rapamycin (which blocks the common pathway of intracellular growth factor signaling by interfering with the postreceptor signal transduction pathways for the IL-2 receptor on the cell surface).

These developments are principally geared towards the prevention of acute rejection since a reduction in the frequency and severity of acute rejection episodes has the potential in improving the long-term survival of the transplanted allografts (although there is no data yet to prove that these agents have improved long term survival). Nevertheless, acute rejection of the allografts remains a principal cause of graft loss in kidney transplantation. With up to 15 % of allograft kidneys lost to rejection in the first year following transplantation, acute rejection, leading to early or delayed graft loss, has become one of the leading causes of end-stage renal disease in developed countries [Schmouder and Kunkel, 1995].

The continuing shortage of organs available for transplantation is well known, this being principally due to the continued reliance on cadaveric donations which has declined in recent years. The pressure on the transplant community will become even more acute with increasing numbers of patients requiring organ transplantation. The UKTSSA report for 1996 showed that the number of patients on the waiting list for a kidney transplant in the UK was over three times greater than the number of transplants performed [UKTSSA Transplant Activity 1996]. This trend is likely to continue and indeed worsen as the steady increase in the number of patients joining the waiting list is not matched by a comparable increase in cadaveric donation, or even worse, a decrease in organ donation as in recent years. Until the introduction of clinically successful xenotransplantation of tissue engineered kidneys, even with the increase in the number of living-related kidney transplantation, cadaveric donors will continue to be the principal source of kidneys for transplantation in the Western world. Therefore, prolonging the duration of useful function for each renal transplant remains the single most effective way of reducing the burden of renal replacement therapy.

To this end therefore, a better understanding of the immunological phenomenon of acute allograft rejection remains an important imperative in the search for improvements in the long-term results of not only renal allograft transplantation, but also other solid organ transplants, in order that such a precious and scarce resource may be maximally utilised.

1.2 Rejection of the allograft following transplantation

Three principal patterns of rejection of the transplanted allografts have been recognised based on clinical presentation and histological criteria. These are, in the order of the time scale of its occurrence, hyperacute rejection, acute rejection and chronic rejection [reviewed in Dallman and Morris, 1994].

Hyperacute rejection, now a rarity, occurs in presensitised recipients within the first few minutes to hours after organ engraftment, often even before the operation has been completed. It is mediated by preformed circulating cytotoxic antibodies directed specifically to the major histocompatibility complex (MHC) antigens expressed by the donor organ resulting from previous exposure to MHC-incompatible tissues (eg. following blood transfusions, pregnancies or previous organ transplantations). Rejection is accompanied by the deposition of antibodies with complement activation within the graft causing endothelial disruption, platelet margination and infiltration by polymorphonuclear leucocytes [Kissmeyer-Nielson et al, 1966; Williams et al, 1968]. The ensuing disseminated thrombosis within the graft results in immediate loss of the graft. However, hyperacute rejection is no longer a problem in renal transplantation, and indeed has become a rare phenomenon nowadays with the routine practice of serological and/or flow cytometric cytotoxic crossmatch prior to transplantation and the increasing practice of antibody characterisation in sensitised patients.

In contrast to hyperacute rejection, acute rejection occurs in naïve recipients, and commonly occurs within the first 14 days after transplantation. Acute rejection can also take place at any time after transplantation if the recipients' immune system was inadequately suppressed by a reduction in immunosuppressive therapy or stimulated by an unrelated event such as a viral infection. As the study of acute allograft rejection is the mainstay of this thesis, this subject will be dealt with in greater detail in the next section.

Chronic rejection is the least characterised and understood form of the allograft rejections. It usually develops over a much longer time scale than acute rejection, probably has a multifactorial aetiology and the histological hallmark is obliterative arterial changes and interstitial fibrosis caused by progressive microvascular endothelial damage resulting possibly from both antibody-mediated and cell-mediated processes. Although non-immunological factors like age of donor and previous cytomegalovirus infection may influence the development of chronic rejection, the frequency of acute rejection episodes in the early months after transplantation is often a good predictor of the subsequent development of chronic rejection [Dallman and Morris, 1994]. The changes of chronic rejection are often slow and insidious, it is nevertheless relentless and the final outcome is invariably graft failure. There is no therapeutic manipulation currently available that has been shown to be effective in altering the course of chronic rejection [Gibbs, 1997].

1.3 Acute allograft rejection

Acute rejection of a kidney allograft is a complex immunological process. It involves both functional types of the body's immune system, namely, non-specific (or innate) immunity and specific (or acquired) immunity, with considerable interdependence between the different cell subpopulations [Hall, 1991; Schmouder and Kunkel, 1995]. The initial trigger for this complex immunological process commences with the recognition of allogeneic MHC antigens on the allograft tissues by the recipient's

immune system [Hutchinson, 1991]. The principal components of the recipient's immune system mediating this recognition process during acute allograft rejection are the T cells .

1.3.1 Role of the cell surface molecules

The MHC antigens are glycoproteins present on cell membranes that determine the response to tissue allografts between different members of the same species. The MHC in each species is genetically determined, with considerable similarity between the MHC in different species with respect to both the immunogenetics and biochemistry of the different systems [Dallman and Morris, 1994]. In man, the MHC contains the human leucocyte antigen (HLA) system which is subdivided into class I and class II antigens, based on their structure, tissue distribution and function. The 'classical' class I antigens (encoded by the HLA-A, -B and -C loci) each consist of a polymorphic heavy chain (45kd) in association with a non-variable light chain (12kd) - β2 microglobulin - are expressed on virtually all nucleated cells and platelets, and are responsible for activating T cells of the CD8 (largely cytotoxic) phenotype by presentation of peptides derived from antigens mostly of endogenous origin. The class II antigens (of HLA-DP, -DQ and -DR subclasses) are each comprised of two polymorphic chains of similar molecular weight (α chain 35kd and β chain 28kd) and stimulate T cells of the CD4 (mainly helper) phenotype via presentation of peptides derived from antigens largely of exogenous origin. Class II antigens have a more restricted tissue distribution, being constitutively expressed by only B cells, dendritic cells and some endothelial cells. The importance of HLA in renal transplantation has been shown by the better short-term and long-term graft survival data from large collaborative studies like UNOS and CTS registries based on improved HLA-matching for HLA-A, -B and -DR [Morris, 1994].

T cells recognise the antigenic determinants presented by foreign MHC antigens via the interaction of their surface-bound T cell receptors (TCR) and the MHC molecules on the surface of antigen presenting cells (APCs). This cognate interaction between TCR and MHC confers the first of two signals necessary for T cell activation and

takes place in association with CD4 (for MHC class II antigens) and CD8 (for MHC class I antigens) molecules on the surface of T cells. The second, or co-stimulatory signals, which is essential for T cell activation, can be mediated by the interactions between the other cell surface ligands on the T cells and APCs (like the interactions between CD2 and CD58, CD5 and CD72, CD11a/CD18 and ICAM-1, -2, -3, and CD28/CTLA-4 and CD80/B7-1) [Cuturi et al, 1994].

The interaction between TCR and MHC has been shown to take place by two different pathways. In the so-called "direct" pathway (figure 1.3.1), T cells recognize intact allo-MHC molecules, with or without bound peptides, expressed on the surface of donor-APCs, while in the so-called "indirect" pathway (figure 1.3.2), T cells recognize processed peptides of alloantigens presented in the context of the recipient's self-APCs [reviewed in Sayegh et al, 1994]. The indirect pathway is therefore equivalent to that which operate in the normal immune response. These pathways need not be mutually exclusive during the rejection process because each is mediated by different sets of T cell clones. The direct pathway accounts for most of the cytotoxic T cell function, whereas the indirect pathway resulting from processed determinants presented on recipient class II MHC molecules may account for much of the T helper cell function [Sayegh et al, 1994]. The T cell response that will ultimately result in early acute allograft rejection seems to be primarily via the direct recognition of donor MHC molecules. The frequency of T cells that are engaged in the indirect pathway of allorecognition has been estimated at about 100-fold lower than that of T cells participating in direct allorecognition [Liu et al, 1993].

A complex series of events follows allorecognition resulting in the generation of cell-mediated and humoral-mediated effector mechanisms. The cell-mediated immune mechanisms include T cell-mediated processes with high degrees of specificity, as well as more poorly restricted responses by natural killer (NK) cells, lymphokine-activated killer (LAK) cells, and activated macrophages. The humoral-mediated responses can be less-specific natural antibodies or highly specific antibodies of a variety of isotypes that can mediate different functions, including opsonisation, complement activation, antibody-dependent cell-mediated cytolysis,

and neutrophil, eosinophil, or basophil activation [Hall, 1991]. These effector mechanisms, if left unchecked, will ultimately lead to graft destruction.

Figure 1.3.1 – Direct T cell recognition of allo-MHC (reproduced with permission from Sayegh et al, 1994) – recipient's T cells can recognize empty MHC molecules (top) or allo-MHC + peptide complex (bottom). These peptides could be derived from endogenous proteins or from donor or recipient MHC.

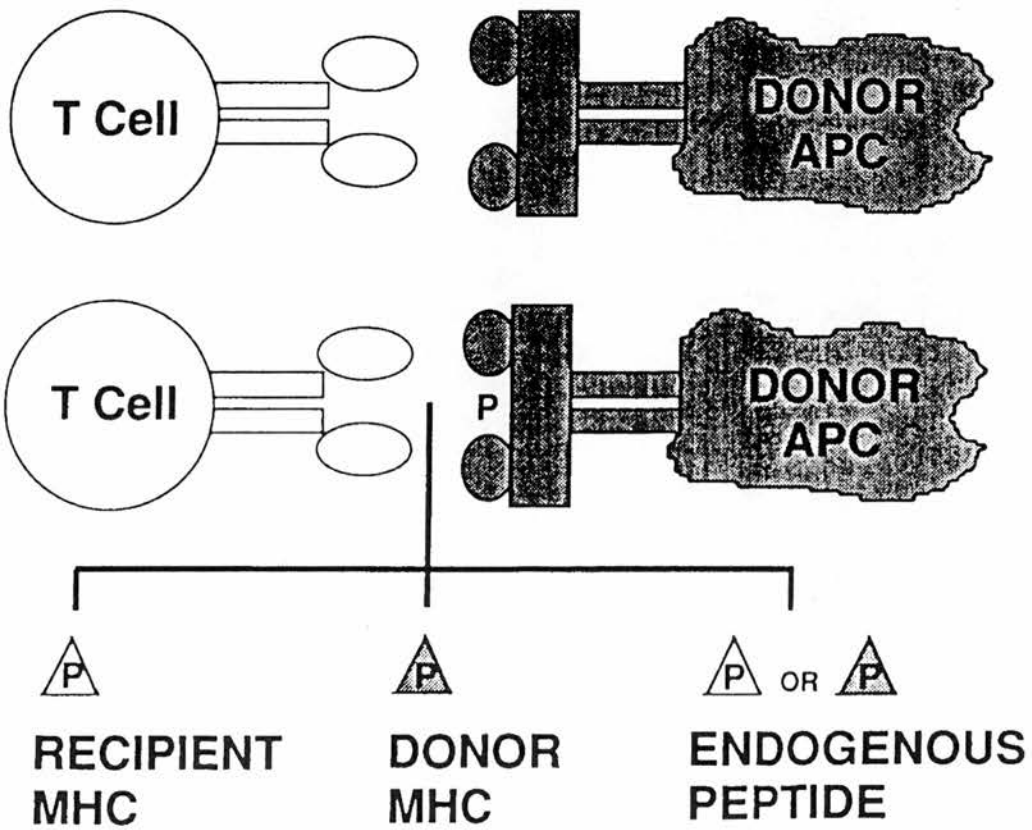
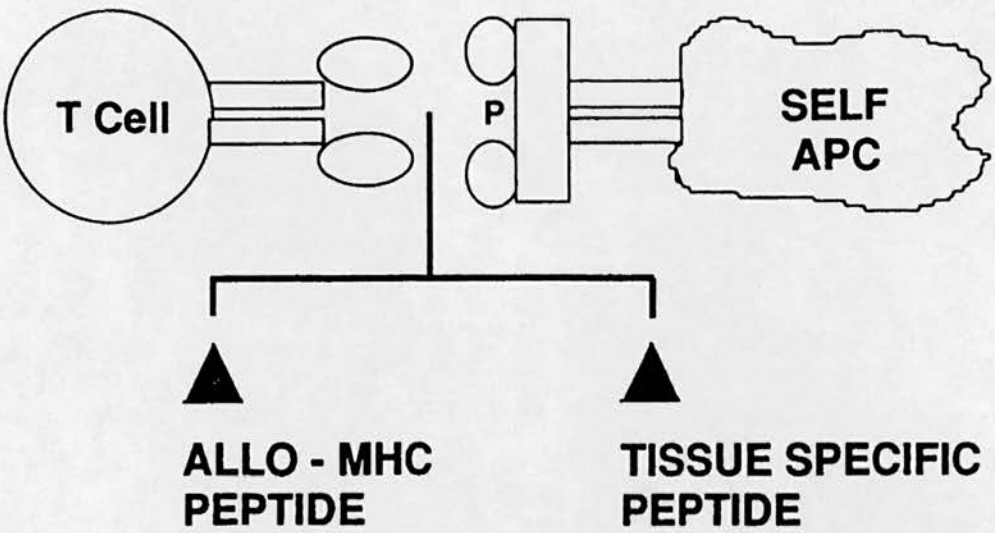


Figure 1.3.2 – Indirect self-restricted T cell recognition of alloantigen (reproduced with permission from Sayegh et al, 1994) – recipient's T cells recognise allopeptides bound to self-MHC. These peptides are predominantly processed donor MHC molecules, although some may be derived from tissue-specific peptides.



1.3.2 Role of cytokines

The complexity and unpredictability of acute renal allograft rejection points to the involvement of multiple signals which initiate, modulate and effect this process. These important signals of intercellular communications [Dallman et al, 1991] are now recognised as soluble endogenous biological products of cells, called cytokines, a general term which refers to many different immune signal peptides like lymphokines, chemokines, interleukins and colony stimulating factors. Indeed, it is now clear that the ability of a T cell to respond to a given alloantigen is determined not only by the TCR and other ligands expressed on their cell surface, but by their capacity to produce these cytokines to promote their growth and function.

In contradistinction to hormones which acts systemically, cytokines are only active in the local cellular microenvironment, serving as the medium of intercellular communication between neighbouring cells a short distance away from the cytokine

source via both paracrine and autocrine means. Both immune and non-immune cells are capable of secreting these different cytokines under various stimulatory conditions.

Upon their release, cytokines can activate their target cells by binding to their specific surface-bound cytokine receptors on the target cells. The end result of the cytokine action can be the secretion of other cytokines; differentiation, upregulation or downregulation of the target cells; or feed back upon the cell of origin inducing further secretion of that particular cytokine. Consequently, a cascade of inflammatory signals is created via these regulatory networks of positive and negative interactions to produce the histological lesions characteristic of acute allograft rejection. These characteristic lesions include the vascular abnormalities of vasodilatation, of increased vascular permeability with augmented adherence of blood cells to vascular endothelium leading to the infiltration of the rejecting allograft by inflammatory and immune cells; and an increased pro-coagulant state resulting in thrombosis, haemorrhage and eventually destruction of vessels [Chatenoud, 1992].

Current knowledge of the role of cytokines in acute allograft rejection is still rather limited with most of the laboratory observations from in vitro and animal experiments. Since cytokines are released locally into the cellular microenvironment and their activities are transient in nature, the relevance of systemic measurement of cytokine protein products in order to predict graft rejection is therefore rather limited. Moreover, elevated cytokine product levels are non-specific indicators of the presence of an acute inflammatory response since they have been found both during rejection and infection [Vossen and Savelkoul, 1994]. Studies into the role of cytokines in acute allograft rejection have therefore moved from the analysis of the cytokine products themselves to the detection of their gene expression within the transplanted allografts.

1.3.3 Cytokine gene expression in acute rejection

In the last few years, several studies looking at cytokine gene expression have linked the expression of many different cytokines with acute rejection in different human organ transplants. Most of these were single time-point studies in that cytokine gene transcripts were only studied at the time of rejection and were not correlated with the events in the grafts prior to the acute rejection episode or following anti-rejection therapy. It is therefore not surprising that despite these numerous studies, there has been no overall concensus as to which cytokines are truly indicative or predictive of acute cellular rejection, or its corollary, tolerance. An overview of the literature concerning the relevance of some of these key cytokines in acute allograft rejection is detailed in section 1.5.

1.4 The T-helper (Th) 1/Th2 Paradigm

The discovery that T-helper lymphocytes in murine T-cell clones can be classified into two groups based on their phenotypically distinct cytokine profiles with different functional properties [Mosman et al, 1986, Mosmann and Coffman, 1989] has resulted in the evolution of a unifying concept to explain the observed cytokine profiles in studies elucidating the cytokine programs associated with T cell activation, allograft rejection and tolerance induction, the so-called Th1 and Th2 paradigm [Nickerson et al, 1994].

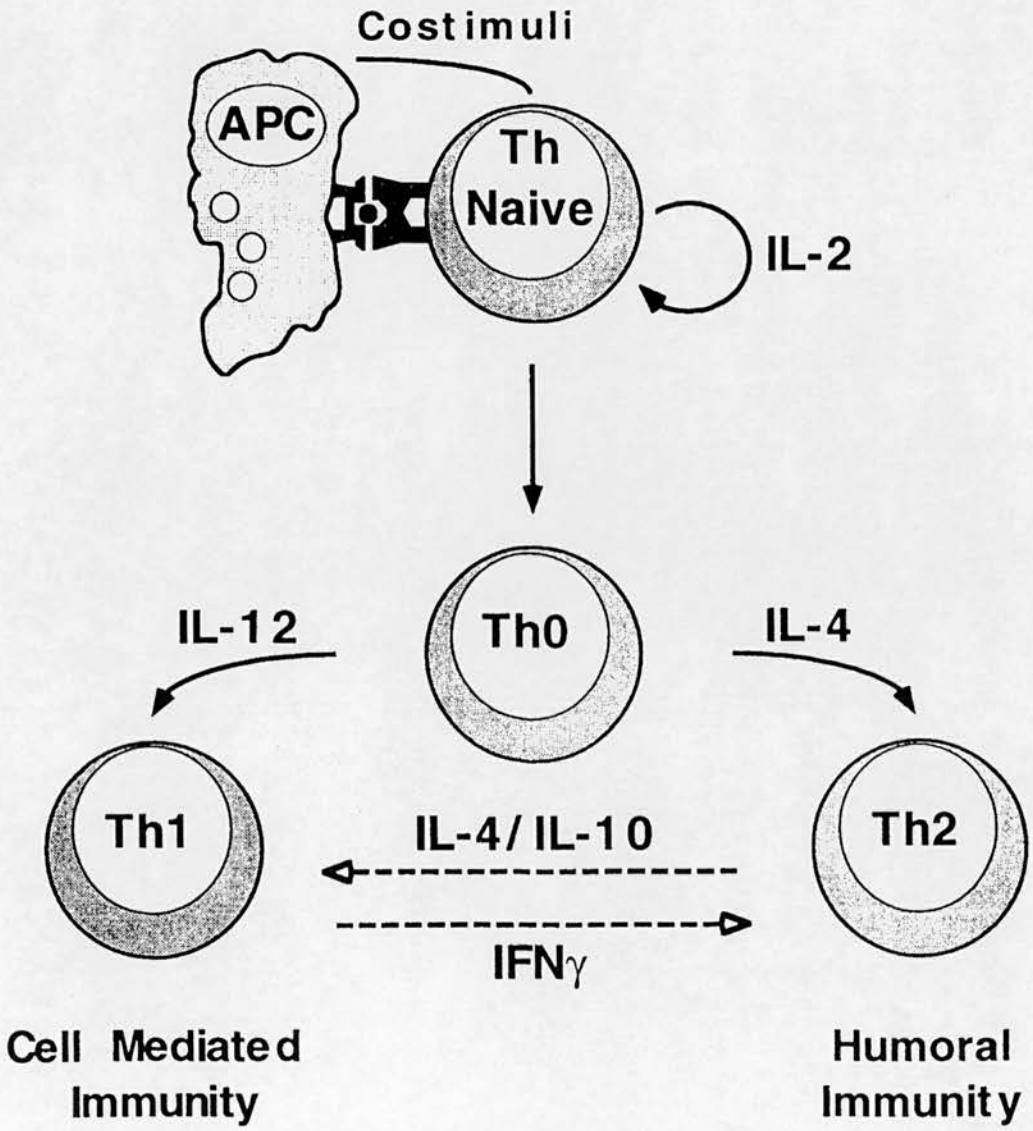
$CD4^+$ cells of the Th1 subset are strongly associated with cell-mediated immunity, producing interleukin (IL)-2, interferon-gamma (IFN-γ) and lymphotoxin (also called tumour necrosis factor-beta, TNF-β) on activation, while $CD4^+$ cells of the Th2 subset are often associated with humoral immunity, producing IL-4, IL-5, IL-6, IL-10 and IL-13 [Nickerson et al, 1994; Mosman and Sad, 1996].

Both Th1 and Th2 cells developed from a common precursor, the Th0 cells (producing IL-2, IL-4, IFN-γ and TNF-β), and the commitment towards the

development of either Th1 or Th2 subsets depends on the presence of exogenous IL-12 or IL-4 respectively [Nickerson et al, 1994]. Th0 cells differentiate from activated naïve T cells in the presence of IL-2 following the complex event of allorecognition described in the previous section. The two Th subsets cross-regulate Th1/Th2 function by the release of their individual cytokines into the cellular microenvironment, the central theme to this paradigm [Fiorentino et al, 1989; Fitch et al, 1993]. For example, IFN-γ, a Th1 product, inhibits expression of the Th2 program while IL-4 and IL-10, both Th2 products, can act to block the Th1 program. Hence, activation of the Th1 phenotype is often accompanied by silencing of the Th2 program and vice versa [Nickerson et al, 1994]. Figure 1.4.1 summarizes the interaction between the various T helper cells.

It is widely believed that Th1 cells play an important role in the allograft rejection process since Th1-type immune activation is usually manifested during allograft rejection [Strom et al, 1996]. Since Th2 cells are antagonistic to Th1 development and function, a popular hypothesis states that preferential induction of allograft-specific Th2 cells would down-regulate Th1-driven rejection responses, thereby promoting allograft acceptance or tolerance [Lowry and Takeuchi, 1996; Piccotti et al, 1997]. While this hypothesis is supported by a number of studies demonstrating the preferential activation of cells producing Th2 cytokines (IL-4) in conjunction with the absence/reduction of cells producing Th1 cytokines (IL-2) in allograft hosts receiving tolerizing therapies [Mohler and Streilein, 1989; Powell and Streilein, 1990], the picture is not entirely clear with several preclinical models showing the presence of Th2 response to accompany rejection [Strom et al, 1996]. The question of whether Th2 cells are beneficial, deleterious, or irrelevant in promoting allograft survival was recently reviewed by Piccotti and colleagues [1997], and their conclusion was that in the context of transplantation, Th2 cells may indeed be beneficial, deleterious, and/or irrelevant! The confusing messages emanating from the cytokine literature regarding the role of Th1 and Th2 cytokines in the allograft response are reviewed in the following section.

Figure 1.4.1 – T cell activation and cross-regulation (reproduced with permission from Nickerson et al, 1997) – solid arrows indicate positive effect, dashed arrows indicate inhibitory effect.



Therefore, the Th1/Th2 paradigm while useful in explaining the observed cytokine profiles in animal models, has not been as clear in human studies, even though the Th1 and Th2 patterns of cytokine production have also been described among human T cells [Del Prete et al, 1991]. Moreover, recent studies highlighting the redundancy

of the immune system (both in term of the variety of cytokines able to act as T cell growth factors, and in the use of common cytokine receptor subunits for signal transduction by groups of cytokines) and the pleiotropic nature of the cytokine network (in term of the numerous functions that any one cytokine can perform) indicate that the Th1/Th2 paradigm in itself may not be sufficient to explain fully the mechanisms underlying rejection and tolerance in transplantation [Nickerson et al, 1994].

Hence, it has become apparent that the Th1/Th2 paradigm, while providing a valuable framework for probing various models, has undoubtedly oversimplified the complexity of cytokine responses by individual T cells in vivo. Indeed, because cytokines are pleiotropic, the microenvironment in which they are released may be the determining factor whether the effects of a cytokine are immunoregulatory or proinflammatory [Nickerson et al, 1997]. It has also been suggested that Th1 and Th2 cells may not, after all, be discrete cellular compartments in the immune systems but rather two extreme endpoints in a continuous spectrum, and the two patterns of cytokine expression may simply represent extremes of many possible outcomes [Kelso, 1995; Mosmann and Sad 1996].

1.5 Review of the literature on cytokines and cytotoxic T lymphocyte (CTL) markers studied in the project

The Th1 cytokines studied in the project were IL-2 and IFN-γ, and the Th2 cytokines studied were IL-4, IL-5, IL-10 and IL-13. The reason why more Th2 cytokines were studied than Th1 cytokines was because following the first batch of gene expression experiments looking at IL-2, IFN-γ, IL-4 and IL-10, we have found much more interesting and significant changes in the level of the two Th2 cytokines as compared with Th1 cytokines in the project's series of patients. Therefore, additional Th2 cytokines were studied in the latter part of the project.

As the detection of other early activation markers of the immune system (apart from cytokine profiles) had been published in the recent literature [Suthanthiran, 1997; Strehlau et al, 1997], we chose to examine two of these cytotoxic T lymphocyte (CTL) activation markers, namely granzyme B (GrB) and fas ligand (FasL), at the same time points as for the cytokines and correlate their expression with the clinical events.

Despite extensive literature into the roles cytokine play in the underlying immunobiology of acute rejection in solid organ transplantation, there is still much confusion about the exact role and relative importance of individual Th1 and Th2 cytokines. The complex nature of the cytokine response to the presence of the allograft during an acute rejection process, coupled to the confounding effect of the different methods of measuring the cytokine response, have undoubtedly contributed to many of the conflicting findings that have been published in cytokine research literature [Baan and Weimar, 1998].

The following sub-sections review the literature on the evidence, or otherwise, for the role in acute allograft rejection of the individual cytokines and CTL activation markers studied in the project.

1.5.1 IL-2

IL-2 was discovered through its activity as a T cell growth factor (TCGF) [Gillis et al, 1978] and is secreted and synthesized by activated T cells [reviewed in O'Garra, 1989a]. It interacts with the binding site residues of its IL-2 receptor complex (IL-2R), an $\alpha\beta\gamma$ heterotrimer of distinct polypeptide chains [Takeshita et al, 1992], resulting in a high-affinity binding. By its principal activity as a TCGF, IL-2 stimulates the proliferation of $CD4^+$ T cells (by clonal expansion of alloantigen-activated T cells via autocrine stimulation) as well as activating $CD8^+$ CTLs and LAK cells [O'Garra, 1989a; Nickerson et al, 1994]. It also stimulates NK cell proliferation and activation [O'Garra, 1989a; Chatenoud, 1992; Schmouder and Kunkel, 1995]. It induces the production of other proinflammatory cytokines by

activated NK cells (IFN-γ and TNF-α) [Anegon et al, 1988] and macrophages (TNF-α) [Economou et al, 1989], thus amplifying the acute inflammatory response. Under some conditions, B cells may also be induced by IL-2 to proliferate and differentiate into antibody-secreting cells [Mingari et al, 1984; Muraguchi et al, 1985], and this effect may be enhanced by IFN-γ [Nakagawa et al, 1985].

As one of the main proinflammatory cytokine products of Th1 cells, IL-2 has always been seen as a central player in the allograft rejection process. It has been shown that the administration of IL-2, as an adjunct to tolerizing therapies, precludes engraftment [Dallman et al, 1991c]. IL-2 protein products (detected directly or following stimulation of peripheral lymphocytes) in plasma or urine have been found to be elevated in patients experiencing acute rejection following renal transplantation [Vie et al, 1985; Simpson et al, 1989; Colvin et al, 1990]. Some authors have found that elevated plasma IL-2 predicts impending rejection [Kutukculer et al, 1995], while others have found that although plasma IL-2 was significantly higher in patients who experienced acute rejection, it was not sufficiently reliable for diagnosis or exclusion of rejection [Johnson et al, 1990].

This association of IL-2 with allograft rejection is also seen in several animal studies of cytokine gene expression [Dallman et al, 1991a; Papp et al, 1992; Takeuchi et al, 1992; Wu et al, 1992; O'Connell et al, 1993] and immunocytochemistry [Mottram et al, 1995; Sayegh et al, 1995]. In the clinical situation, similar associations were often reported in renal transplants [Xu et al, 1995; Kirk et al, 1995; Suthanthiran et al, 1997], liver transplants [Gaweco et al, 1995] and heart transplants [Baan et al, 1994]. In a sequential analysis of intragraft IL-2 gene transcription in a small group of renal transplant patients, Dallman et al [1992] found that IL-2 gene expression appeared at a very early stage of the immune response to the graft, before acute rejection was detectable by clinical or biochemical changes. The presence of IL-2R in renal allografts was also found to be a significant correlate of acute cellular rejection [Noronha et al, 1992].

Indirect evidence for the importance of IL-2 in allograft rejection is seen by a decrease in peripheral IL-2 products with anti-rejection treatment [Daniel et al, 1995] and in experimental models of peripheral tolerance showing a lower level of intragraft IL-2 gene expression in tolerant animals than in rejecting ones [Bugeon et al, 1992; Takeuchi et al, 1992]. Further indirect evidence supporting the role of IL-2 in the rejection process is also seen from the success of using monoclonal antibody to IL-2 or IL-2R in prolonging allograft survival by preventing and/or reversing rejection in experimental [Kirkman et al, 1985; Kupiec-Weglinski et al, 1986; Sakagami et al, 1987] and clinical transplantation [Soulillou et al, 1990; Kirkman et al, 1991].

However, while the association of IL-2 with allograft rejection may be important and compelling, the presence of IL-2 is nevertheless non-specific as the Oxford group had reported that proinflammatory cytokines like IL-2 and IFN-γ may be "necessary but not sufficient for the development of acute cellular rejection". Indeed, they found that in non-rejectors, the patterns of these cytokines in the allografts were indistinguisable from the rejectors [McLean et al, 1997]. Experiments using IL-2 knock-out mice (mice whose IL-2 gene has been functionally silenced by targeted gene disruption) have shown that allograft rejection readily occurs in the absence of IL-2 [reviewed by Nickerson et al, 1994].

At the same time, there have been many studies which failed to detect and/or associate the presence of intragraft IL-2 gene expression with rejection episodes in human cardiac transplants [Wu et al, 1994], liver transplants [Martinez et al, 1992; Martinez et al, 1993b] and renal transplants [Krams et al, 1992; Jeyarajah et al, 1995; Strehlau et al, 1996; Strehlau et al, 1997]. The reasons cited for the lack of expression of IL-2 in these studies are that this cytokine gene is only expressed transiently upon activation [Krönke et al, 1984; Wiskocil et al, 1985; Shaw et al, 1988], or it is expressed early in the rejection process and therefore not observed in biopsies during overt rejection episodes [Martinez et al, 1992; Dallman, 1993], or the failure to detect IL-2 gene expression is the result of inhibition by cyclosporin [Krönke et al, 1984; Wiskocil et al, 1985; Wu et al, 1994]. It is also well documented

that the IL-2 message rapidly disappears with the removal of the stimulus [Shaw et al, 1988; Swoboda et al, 1991].

The value of peripheral cytokine protein product measurement has always been controversial. Since cytokines only act locally within the cellular microenvironment by autocrine or paracrine action, and together with their short half-life, the interpretation of their peripheral levels is difficult, thus making its value questionable [McKenna et al, 1988]. One group evaluating cytokines and cytokine-induced secondary messages in sera of patients following liver transplantation has come to the same conclusion that the clinical usefulness of measuring cytokines is limited [Tilg et al, 1990]. They also found that acute rejection was not accompanied by the preferential enhancement of IL-2 or IFN-γ, indeed IL-2 was not detectable at all in peripheral blood.

1.5.2 IFN-γ

This cytokine, also called macrophage activating factor (MAF), is produced by antigen-specific T cells during an immune response, by NK cells recruited by IL-2 [reviewed in O'Garra, 1989b], and by eosinophils [Lamkhioued B et al, 1996]. It recruits and activates macrophages (by enhancing phagocytosis and tumour killing capability) and enhances growth and activation of CTLs and NK cells [Trinchieri and Perussia, 1985]. One of its most important activities is the upregulation of MHC class I (widely distributed on all nucleated cells except foetal cytotrophoblast tissue [Colvin, 1990]) and class II (normally expressed at high density by APCs like dendritic/Langerhans cells, macrophages and B cells, human capillary endothelium, and activated T cells [Colvin, 1990]). These activities of IFN-γ, together with its induction of the de novo synthesis of MHC class II antigens on cells which do not constitutively express them and the expression of high-affinity Fc receptor for monomeric IgG on myelo-monocytic cells [Trinchieri and Perussia, 1985; Gerrard et al, 1988], have the effect of making the tissues more immunogenic. IFN-γ regulates humoral immune responses by inducing immunoglobulin secretion by activated B cells stimulated by IL-2 [Nakagawa et al, 1985; Lê thi Bich-Thuy et al, 1986] and

potentiates IL-4-induced proliferation of B cells [Defrance et al, 1987]. It selectively induces high levels of complement-fixing cytotoxic IgG_{2a} antibody [Snapper et al, 1988a] and inhibits many of the activity of IL-4 [Rabin et al, 1986], which could influence the proliferation of Th2 cells. Hence IFN-γ and IL-4 reciprocally regulate the immunoglobulin isotype production by B cells in T cell-dependent immune responses [Coffman and Carty, 1986; Snapper et al, 1988a]. IFN-γ also upregulates the expression of ICAM-1 on many cell types, including endothelial cells and fibroblasts [Dustin et al, 1986; Rothlein et al, 1988], thus indirectly mediating T cell antigen-specific responses and their migration to sites of inflammation.

Like IL-2, IFN-γ is a prominent member of the proinflammatory Th1 family of cytokines and consequently it has also been seen by many as another key player in the allograft rejection process. Not surprisingly, therefore, several studies in experimental and clinical transplantation which associated IL-2 with rejection also implicated IFN-γ [Wu et al, 1992; O'Connell et al, 1993; Mottram et al, 1995; Kirk et al, 1995; Sayegh et al, 1995; Thai et al, 1995]. In addition, a number of other studies looking at stimulated cytokine products [Benvenuto et al, 1991; Merville et al, 1993; Kaminski et al, 1995], in situ cytokine expression [Noronha et al, 1992] and cytokine gene expression [Nast et al, 1994; Zuo et al, 1995] also associate IFN-γ with allograft rejection.

Indirect evidence suggesting a role for IFN-γ in the mechanisms underlying allograft rejection is seen in experimental models of peripheral tolerance where intragraft IFN-γ expression was reduced by more than 90% in grafts from tolerant recipients [Takeuchi et al, 1992] and a lower level of IFN-γ gene transcripts accumulated within the grafts with delayed kinetics (i.e. a slower rate of rise in intragraft IFN-γ) is seen in tolerant grafts compared with rejected ones, and that treatment of the animals with IFN-γ abrogates the induction of tolerance in the recipients receiving the tolerizing regime of pretransplant donor blood transfusion [Bugeon et al, 1992]. In a more recent study, the co-injection of IFN-γ at the time of alloimmunization to induce neonatal tolerance in mice restored the ability of the neonatal primed mice to reject donor-bearing skin grafts, leading to the conclusion that the prevention of Th1

responses that lead to rejection is critical for experimental tolerance induction [Chen et al, 1996].

As mentioned in the previous section on IL-2, McLean et al [1997] have concluded that IFN-γ, like IL-2, may be "necessary but not sufficient for the development of acute cellular rejection" as the sequential patterns of IFN-γ gene expression in rejectors were indistinguisable from that found in non-rejectors in the first 10 days following kidney transplantation. As for IL-2, workers have found that IFN-γ knockout hosts reject heterotopic heart transplants with a time course and histologic pattern similar to those of the wild-type host, and expression of other proinflammatory cytokines noted during the allograft response in IFN-γ knockout mice is unchanged [Konieczny et al, 1996]. Similarly, islet allograft rejection in IFN-γ receptor knockout hosts is brisk and T cell dependent [Steiger et al, 1996].

Several other studies examining the level of cytokine products following liver [Tilg et al, 1990] and renal [Kutukculer et al, 1995] transplantation, and gene expression studies following renal transplantation [Vandenbroecke et al, 1991; Krams et al, 1992; Xu et al, 1995] also failed to associate the presence of IFN-γ with allograft rejection. Failure to detect IFN-γ in some of the studies was blamed on early or transient expression or suppression by cyclosporin A, as for IL-2 as described previously.

1.5.3 IL-4

IL-4, previously called B-cell stimulatory factor-1, was originally discovered as a costimulator of B cell proliferation [Howard et al, 1982], but it has since been shown to act on a wide variety of cell types including T cells, macrophages, mast cells and several haematopoietic lineage cells [Paul and Ohara, 1987]. It is produced by the Th2 subset of activated T cells [O'Garra, 1989b] and eosinophils [Lamkhioued B et al, 1996]. It stimulates resting T cells to proliferate [Hu-Li et al, 1987] and is considered to be the central autocrine growth factor that drives the development of Th2 cells [Swain et al, 1990]. Its principal activity, however, is as B cell growth

factor, causing clonal expansion of antigen-specific B cells [reviewed in O'Garra, 1989b]. It is also very important in the regulation of immunoglobulin isotype switching in uncommitted B cells, inducing the production of IgG_1 isotype and high levels of IgE [Bergstedt-Lindqvist et al, 1988; Snapper et al, 1988b], thus modulating the humoral responses to different antigenic stimuli.

The other proinflammatory activities of IL-4 include its ability to: (a) selectively induce resting B cells to increase their expression of MHC class II antigens [Noelle et al, 1986] and B7 costimulatory ligands [Stack et al, 1994], thus enhancing the ability of B cells to present antigens and making the immune system more sensitive to smaller amounts of antigens; (b) increase chemoattraction of macrophages [Hiester et al, 1992]; (c) increase expression of adhesion molecules VCAM-1 on vascular endothelium [Masinovsky et al, 1990; Thornhill et al, 1991] and ICAM-1 on interstitial fibroblasts [Piela-Smith et al, 1992] either on its own or synergistically with other cytokines, thus facilitating transmigration of immune cells; (d) induce both proliferation and cytolytic activity of CTL [Widmer and Grabstein, 1987].

On the other hand, some of the other effects of IL-4 can be classified as being immunosuppressive. For example, it antagonises many activities mediated by IFN-γ [Mossman and Sad, 1996], and together with its downregulatory effect on IL-2R and blocking of IL-2-dependent proliferation of T cells [Martinez et al, 1990], IL-4 indirectly inhibits the development and function of Th1 cells. It downregulates IFN-γ production by human peripheral blood mononuclear cells [Peleman et al, 1989], inhibits the IFN-γ-induced production of IgG_{2a} [Rabin et al, 1986] and inhibits IL-2-induced proliferation of B cells [Defrance et al, 1988; Jelinek and Lipsky, 1988; Karray et al, 1988] and LAK cells [Spits et al, 1988]. IL-4 is also a potent inhibitor of macrophage functions mediated by IFN-γ, reducing their production of TNF-α, IL-1, prostaglandin E_2 [Essner et al, 1989; Hart et al, 1989], and reactive oxygen intermediates [Lehn et al, 1989].

As one of the principal Th2 cytokines, IL-4 has in general been associated with the induction and/or maintenance of tolerance following experimental allograft

transplantation [Mohler and Streilein, 1989; Powell and Streilein, 1990; Takeuchi et al, 1992; Donckier et al, 1995; Mottram et al, 1995; Sayegh et al, 1995] and tolerance fails in the absence of IL-4 [Schurmans et al, 1990]. However, in a recent review of tolerance induction in experimental transplantations, Nickerson et al [1997] concluded that IL-4 on its own does not lead to tolerance nor does the absence of IL-4 preclude permanent engraftment.

Indeed, IL-4 gene expression had been associated with allograft rejection in different clinical organ transplantations [Krams et al, 1992; Whitehead et al, 1993; Gaweco et al, 1995; Lang et al, 1995; Lang et al, 1996], and in several other experimental models of rejection/tolerance where expression of both IL-4 and IFN-γ occurred with rejection [Dallman et al, 1991a; Papp et al, 1992; Chan et al, 1995; Chen et al, 1996]. Enhanced intragraft IL-4 expression was found during acute xenograft rejection and it was selectively enhanced in induced rejection of long-term functioning xenograft in mice [Morris et al, 1995]. Nevertheless, in other animal [O'Connell et al, 1993] and clinical studies [Xu et al, 1995; Strehlau et al, 1996; Strehlau et al, 1997], IL-4 was found not to be associated with allograft rejection. Likewise, the presence of IL-4 cytokine products by direct assay [Kutukculer et al, 1995] or following stimulation of isolated peripheral mononuclear cells [Kaminski et al, 1995] have reached opposite conclusions with regard to the role of IL-4 in acute rejection.

1.5.4 IL-10

IL-10 was initially described as a murine Th2 cell product which inhibited cytokine synthesis by Th1 cells and was originally called cytokine synthesis inhibitory factor because of its inhibitory in vitro effects on a number of immune assays [Fiorentino et al, 1989]. However, IL-10 is not a typical Th2 cytokine as it is synthesized by other T cells (including CD4+ Th0 and Th1 cells, and CD8+ T cells) [Yssel et al, 1992], B cells [O'Garra et al, 1990; Vieira et al, 1991; O'Garra et al, 1992], antigen-presenting cells (monocytes/macrophages) [de Waal Malefyt et al, 1991b; Fiorentino et al, 1991a], eosinophils [Lamkhioued B et al, 1996], various tumour cell lines, including melanomas and ovarian and other carcinomas [Yssel and de Waal Malefyt, 1995] and

keratinocytes [Enk and Katz, 1992]. Indeed, it has been reported recently that $CD14^+$ monocytes/macrophages are the dominant source of human IL-10 [Hagiwara et al, 1995], serving an autoregulatory role [de Waal Malefyt et al, 1991b; de Waal Malefyt et al, 1995] and very recently, it has also been shown that human NK cells produced IL-10 following stimulation with IL-2 [Mehrotra et al, 1998].

IL-10 is a pleiotropic cytokine and modulates the function of T cells, B cells, NK cells, monocytes/macrophages, mast cells and neutrophils [Moore et al, 1993; Yssel and de Waal Malefyt, 1995; de Waal Malefyt et al, 1995]. Although the effect of IL-10 on the expression of other cytokines has suggested that it promotes Th2 cells and inhibits Th1 cells, cytokines and related immune phenomena, these effects are by no means strictly compartmentalised, as human Th1 and Th2 clones can both produce and be inhibited by IL-10 [Del Prete et al, 1993]. This is especially true in the context of transplantation, where IL-10 has been seen as an immunosuppressive cytokine although conflicting evidence prevailed [Bromberg, 1995].

Most of the activities of IL-10 can be classed as being immunosuppressive. IL-10 indirectly prevents antigen-specific T cell activation and proliferation by its action on APC, via the inhibition of antigen presentation and accessory cell functions of APC [Fiorentino et al, 1991b; Enk et al, 1993; Moore et al, 1993; de Waal Malefyt et al, 1995] such as the production of IL-12 and TNF-α [D'Andrea et al, 1993; Trip et al, 1993], costimulatory B7 ligand expression [Ding et al, 1993], ICAM-1 expression [Chang et al, 1994], and the downregulation of MHC class II molecules [de Waal Malefyt et al, 1991a]. IL-10 also inhibits T cell activation and proliferation directly via its inhibitory effects on the production of IL-2 and IFN-γ by the responding T cells [Del Prete et al, 1993; de Waal Malefyt et al, 1993a]. The other monocyte-derived cytokines that IL-10 suppresses are IL-1, IL-6, IL-8, granulocyte-macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF) [de Waal Malefyt et al, 1991b; Fiorentino et al, 1991a]. IL-10 also prevents macrophage cytotoxic activity by suppressing the release of reactive oxygen intermediates [Bogdan et al, 1991] and nitric oxide production [de Waal Malefyt et al, 1995]. A further immunosuppressive property of IL-10 is

seen via the blockade of the proinflammatory activities of IL-1 by IL-1 receptor antagonist (IL-1ra) released from monocytes and polymorphonuclear leucocytes [Cassatella et al, 1994; Jenkins et al, 1994] which is upregulated by IL-10. Recent evidence suggests that IL-10 may also have a role in suppressing Th2-mediated allergic inflammatory processes both directly by its effects on eosinophils and indirectly by its inhibition of IL-5 synthesis by resting $CD4^+$ T cells and by differentiated Th0 or Th2 clones [reviewed in Pretolani and Goldman, 1997].

Conversely, some actions of IL-10 are immunostimulatory in nature, such as its support for the growth and differentiation of mast cells in the presence of IL-3 or IL-4 [Thompson-Snipes et al, 1991], and enhancing B cell growth, immunoglobulin production [Rousset et al, 1992] and MHC class II expression [Go et al, 1990]. IL-10 stimulated or potentiated the cytolytic activity of LAK cells [Schwarz et al, 1994] and serves as a growth cofactor for immature and mature T cells in the presence of IL-2 and IL-4 [MacNeil et al, 1990]. It is also a specific chemoattractant for human $CD8^+$ T cells, since it lacks chemotactic activity towards $CD4^+$ T cells, monocytes or neutrophils [Jinquan et al, 1993]. IL-10 has been reported to promote the survival of IL-2-dependent T cells otherwise destined to die by apoptosis [Taga et al, 1993], thus prolonging proinflammatory influence of these alloreactive T cells.

The evidence pointing to the important role that IL-10 plays in inducing long-term alloantigen-specific T cell unresponsiveness in vitro, and its possible contribution to the induction and maintenance of transplantation tolerance are accumulating [Roncarolo, 1995; Roncarolo et al, 1996]. As for IL-4, some models of experimental tolerance have also associated IL-10 in the induction and maintenance of tolerance [Takeuchi et al, 1992; Mottram et al, 1995; Sayegh et al, 1995]. Other supportive evidence that IL-10 is directly involved in the process of inducing tolerance and maintaining the tolerant state, and that its presence is not merely an epiphenomenon, come from experimental transplantation models where IL-10 function was deliberately introduced, by adoptive transfer of a Th2-like cell line into mice receiving MHC class II disparate skin grafts, resulting in improved skin allograft survival [Maeda et al, 1994], or blocked with monoclonal antibodies to IL-10,

resulting in accelerated allogeneic skin graft rejection [Gorczynski and Wojcik, 1994].

On the other hand, while some investigators have found that the presence of a high frequency of IL-10-secreting T cells in the graft did not inhibit allograft rejection [Merville et al, 1993; Merville et al, 1995], other workers have found that the systemic administration of IL-10 in a murine cardiac transplantation model resulted in accelerated rejection [Lowry et al 1995; Qian et al, 1996] and that ex-vivo perfusion of donor hearts with IL-10 did not affect subsequent graft survival [Qian et al, 1996]. Other cytokine gene expression studies in clinical liver [Bishop et al, 1993] and renal [Xu et al, 1995; Strehlau et al, 1997; Suthanthiran, 1997] transplantation have associated the presence of IL-10 with acute allograft rejection. One group studying cytokine mRNA profile during acute rejection in renal transplantation found that while IL-10 was not detectable in the biopsies, there was a decrease in the level of IL-10 in the peripheral blood lymphocytes following successful anti-rejection treatment [Jeyarajah et al, 1995].

Conflicting findings are also seen in the ELISA measurement of IL-10 itself in different clinical organ transplantation situations, with one group finding a significant elevation of IL-10 in bile during rejection [Lang et al, 1995; Lang et al, 1996] while another group found that successful treatment of rejection in renal transplant patients appears to induce IL-10 secretion [Daniel et al, 1995].

1.5.5 IL-5

IL-5 is another product of activated Th2 cells [Mosmann and Coffman, 1989] and eosinophils [Lamkhioued B et al, 1996]. Originally called T cell replacement factor and initially described as a factor that induces terminal differentiation of late-developing B cells to Ig-secreting cells [Takatsu et al, 1988], the IL-5 cDNA has been cloned in both mouse and human [Kinashi et al, 1986; Azuma et al, 1986]. IL-5 is a growth and differentiation factor for murine B cells [Harada et al, 1985; Swain, 1985; Takatsu et al, 1988], but its effect on human B cells is more controversial

[Sanderson et al, 1988]. IL-5 may promote the generation of CTL [Takatsu et al, 1987] and IL-2-mediated LAK cell activity [Aoki et al, 1989]. IgA and IL-4-induced IgE synthesis by B cells are enhanced by IL-5 [Yokota et al, 1988]. Finally, IL-5 has profound effects on human eosinophil activation and function [Sanderson et al, 1985; Lopez et al, 1988; Sanderson et al, 1988; Fujisawa et al, 1990], maintaining eosinophil viability, inducing superoxide anion production by eosinophils, and possessing eosinophil chemotactic properties [Yamaguchi et al, 1988].

The principal function of IL-5 that is of interest in transplantation is through its effect on eosinophil activation and function. The role of eosinophils as effector cells in allograft rejection has not been clearly established. Although blood eosinophilia following renal transplantation had been reported to be invariably associated with acute cellular episodes of rejection [Lautenschlager et al, 1985], it had no diagnostic or predictive value for rejection or rejection outcome [Frenken et al, 1987]. Intragraft eosinophilia has also been linked with an adverse outcome following acute renal allograft rejection [Weir et al, 1986; Kormendi and Amend, 1988], but tissue eosinophil count did not differentiate the rejectors from the non-rejectors [Kormendi and Amend, 1988]. Eosinophils have also been shown as one of the principal components in the rejection infiltrate following pig proislet xenograft rejection in mice [Morris et al, 1995]. In clinical liver transplantation, graft eosinophilia has been shown to predict rejection with high sensitivity and specificity [Foster et al, 1989]. Similarly, peripheral eosinophilia has been associated with rejection episodes in renal [Kormendi and Amend, 1988] and liver [Foster et al, 1989] transplantation, and the products of eosinophil degranulation have been detected in renal allograft rejection [Ten et al, 1989].

The association of intragraft expression of IL-5 with allograft rejection has been shown principally by one group of workers in clinical liver [Martinez et al, 1992; Martinez et al, 1993a; Martinez et al, 1993b] and renal transplantation [Krams et al, 1992]. The same group has also found that the level of IL-5 product is specifically elevated in the bile and serum of rejecting liver allograft recipients [Lang et al, 1995]. The link between IL-5 and intragraft eosinophilia with acute allograft rejection has

raised the possibility of a non-classical pathway for allograft rejection mediated by eosinophils [Martinez et al, 1993a]. Only two other group of workers have reported an association of IL-5 with acute allograft rejection, one found a modest increase in IL-5 expression with acute rejection following human lung transplantation [Whitehead et al, 1993], while in another group, IL-5 was readily detectable in rejecting murine cardiac allografts which was accompanied by an influx of eosinophils [Chan et al, 1995].

Most other groups of workers studying cytokine gene expression in different organ transplantation, both experimental and clinical [Dallman et al, 1991a; Wu et al, 1994; Gaweco et al, 1995; Kirk et al, 1995; Jeyarajah et al, 1995], have not found an association of IL-5 with allograft rejection.

1.5.6 IL-13

IL-13 is a cytokine produced by activated Th2 cells, which was first described in mice [Brown et al, 1989], and more recently in humans [McKenzie et al, 1993; Minty et al, 1993]. Activated mast cells produce IL-13 too [Burd et al, 1995]. Like the other Th2 cytokines described previously, IL-13 has both proinflammatory as well as immunosuppressive properties. Indeed, IL-13 has a lot in common with IL-4, not only because of a 20 to 25% amino acid sequence homology, but more importantly in its spectrum of biological activities [McKenzie et al, 1993; Minty et al, 1993], and there is evidence to suggest that they also share a common receptor or receptor component, with the IL-13 receptor appearing to be a functional receptor for IL-4 [Callard et al, 1996].

The proinflammatory actions of IL-13 include the induction of phenotypic changes in human monocytes by the upregulation of adhesion molecules and MHC class II antigen expression thus enhancing its antigen presenting function, and by inhibiting its production of IL-10 [de Waal Malefyt et al, 1993b], a Th2 cytokine widely regarded as an immunosuppressive cytokine. IL-13 has important proinflammatory activities on human B cells by directly modulating their surface phenotype, including

the upregulation of MHC class II antigen expression, besides inducing B cell proliferation, differentiation and immunoglobulin production [McKenzie et al, 1993; Minty et al, 1993; Punnonen et al, 1993]. Moreover, IL-13 acts as an isotype switch factor for IgG_4 and IgE synthesis for both naïve human B cells and immature human B cells derived from fetal bone marrow [Punnonen et al, 1993; Punnonen et al, 1994], although its IgE-inducing activity is independent of IL-4 [Punnonen et al, 1993]. Like IL-4, IL-13 induces VCAM-1 expression in vitro [Bochner et al, 1995], and thus might play an important role in VCAM-1 mediated accumulation of eosinophils, basophils, lymphocytes and monocytes at sites of allergic inflammation. IL-13 also has a small, direct effect on IFN-γ synthesis by large granular lymphocytes, and this effect is enhanced in the presence of IL-2 [Minty et al, 1993].

The immunosuppressive role of IL-13 is demonstrated by its action in decreasing the cytotoxic potential of monocytes/macrophages through the inhibition of antibody-dependent cellular cytotoxicity of human monocytes [de Waal Malefyt et al, 1993b] and decreasing the production of nitric oxide by activated macrophages [Doherty et al, 1993], although this immunosuppression appears to be selective in mice since the phagocytic function of the activated murine macrophages is not affected by IL-13 [Doherty et al, 1993]. Another important immunosuppressive effect of IL-13 is its inhibition of the production of proinflammatory cytokines like IL-1, IL-6 and TNF-α, chemokines like migratory inhibitory factor-1α (MIF-1α) and IL-8, and haematopoietic growth factors like GM-CSF and granulocyte-colony stimulating factor (G-CSF). Moreover by its downregulation of IL-12 and IFN-γ, IL-13 may suppress Th1 cell development and favour the generation of Th2 cells [de Waal Malefyt et al, 1993b].

IL-13 also enhances the production of IL-1ra, which by its blockade of the proinflammatory activities of IL-1, results in further immunosuppression [de Waal Malefyt et al, 1993b]. These immunosuppressive effects of IL-13 are direct and not mediated via endogenous production of IL-10.

Being a relatively recently described cytokine, the role of IL-13 in transplantation immunology remains unknown and has not been systematically investigated in any study published to date.

1.5.7 Granzyme B

Granzyme B (GrB) is a member of a family of serine proteases produced by CTL and NK/LAK cells [Haddad et al, 1990] that in humans includes at least four members [Hameed et al, 1988; Meier et al, 1990]. The mechanisms by which these enzymes exert their toxic effect are not fully understood, although they are clearly involved in CTL-mediated cell lysis since selective serine protease inhibitors can abrogate such activities in vitro [Chang et al, 1980; Redelman and Hudig, 1980; Pasternack et al, 1983; Rodgers et al, 1988]. GrB together with other cytotoxic molecules like perforin, are contained within cytoplasmic granules of CTL [Peters et al, 1989], which supposedly undergo directed exocytosis towards the target cell upon contact with the CTL [Jenne and Tschopp, 1988].

Early studies on clinical renal transplantation [Strom et al, 1975] and experimental cardiac transplantation [Strom et al, 1977] has conclusively demonstrated the presence of functionally active CTLs accumulating within rejecting allografts. More recently, studies from two groups of workers in clinical renal transplantation have found that intragraft [Lipman et al, 1992; Lipman et al, 1994; Sharma et al, 1996; Strehlau et al, 1996; Strehlau et al, 1997; Suthanthiran, 1997] and peripheral blood leucocyte [Vasconcellos et al, 1998] expression of GrB gene transcripts were associated with acute allograft rejection.

1.5.8 Fas ligand (FasL)

The human Fas antigen is a cell surface protein belonging to the tumour necrosis factor/nerve growth factor receptor family mediating apoptosis [Itoh et al, 1991]. Fas ligand is also a membrane protein belonging to the tumour necrosis factor family which is expressed in activated T lymphocytes [Takahashi et al, 1994]. By binding to

its receptor, Fas, FasL induces apoptosis in cells expressing Fas and it is therefore involved in CTL-mediated cytotoxicity [Takahashi et al, 1994].

This CTL activation marker has not been studied extensively in the context of organ transplantation, although there has been a recent upsurge in interest in it. The two groups of workers in clinical renal transplantation who had correlated intragraft [Sharma et al, 1996; Strehlau et al, 1996; Strehlau et al, 1997] and peripheral blood leucocyte [Vasconcellos et al, 1998] GrB gene expression with acute allograft rejection had also found that FasL gene expression was associated with acute allograft rejection. However, while recent studies in clinical liver transplants [Tannapfel et al, 1999], and experimental renal [Wang et al, 1997] and cardiac [Seino et al, 1996; Josien et al, 1998] transplantation have supported the significant contribution of FasL to acute allograft rejection, another study using a murine non-vascularized heterotopic cardiac allograft model has shown that Fas/FasL-mediated cytotoxicity is not required for murine cardiac allograft rejection [Djamali and Odorico, 1998]. Indeed, Tannapfel et al [1999] had found that increased apoptosis, Fas, and FasL expression were, taken by themselves, not useful as indicators of acute rejection and Borson et al [1999] had concluded that while FasL gene expression correlated closely with the rejection of murine skin allografts, FasL was not required for allograft rejection.

1.6 Methods of evaluating the cytokine response following organ transplantation

As was mentioned in the previous section, the monitoring of cytokines in an attempt to shed light on the role they play in acute allograft rejection has progressed from the actual measurement of cytokine protein products to the determination of cytokine gene expression within the allografts or immune cells. This section briefly reviews some of the methods used in monitoring the cytokine response.

Cytokine protein products may be detected in the plasma directly [Johnson et al, 1990; Kutukculer et al, 1995a; Daniel et al, 1995], following stimulation of the isolated peripheral lymphocytes [Vie et al, 1985; McKenna et al, 1988; Kaminski et al, 1995] or graft infiltrating cells [Merville et al, 1993; Yard et al, 1994], or from other body fluids like urine [Simpson et al, 1989; van Oers et al, 1988] and bile [Tilg et al, 1990; Lang et al, 1996]. The advantages of direct measurement of cytokine protein products are the ease of obtaining samples like plasma and other body fluids for measurement, and the assays used to detect the cytokines are familiar techniques of standard ELISA methodology, which have been simplified further by the widespread availability of pre-packed cytokine ELISA kits. However, as cytokines produced within the transplanted allograft are secreted locally into the cellular microenvironment and not directly into the bloodstream, the cytokines detected in plasma or body fluids are therefore considerably diluted out and consequently the failure to detect a particular cytokine in plasma or body fluids may not imply an absence of expression of that cytokine but that the assay has failed to detect it due to the dilutional effect. The detection of stimulated cytokine protein products from isolated peripheral lymphocytes or graft infiltrating cells gets over the problem of the dilutional effect but one needs to be cautious in interpreting results from in vitro stimulation of these cells since the pattern of cytokine release from the cells have been artificially created and may therefore not reflect their in vivo cytokine release patterns.

Cytokine gene expression within the tissues obtained from the allografts may be detected by in situ hybridisation techniques [Vandenbroecke et al, 1991; Grimm et al, 1995], or the level of gene expression may be detected indirectly by the reverse-transcriptase polymerase chain reaction (RT-PCR) technique, as the majority of the studies into acute allograft rejection quoted in the previous section were based on. The techniques for detecting cytokine gene expression gets over the problem of dilutional effect seen in direct measurements of cytokine protein products since the technique is highly sensitive and detects the transcripts present within cells. However, these techniques are technically more demanding, especially the in situ

hybridisation technique. The RT-PCR was the technique used in the present project and will be described in greater detail in the remainder of this section.

The RT-PCR technique is a powerful molecular biological method that can be used to measure cytokine gene transcription in a small number of cells [reviewed in O'Garra and Vieira, 1992]. Basically, the technique is a two-stage process, the first requiring the reverse transcription of the cytokine gene transcripts or messenger ribonucleic acids (mRNAs) into complementary deoxyribonucleic acids (cDNAs), followed by the standard polymerase chain reaction (PCR) to amplify these cytokine cDNAs using two specific oligonucleotide primers that flank each cDNA sequence to be amplified. Repeated cycles of thermal denaturation of the cDNA, annealing of the primers to their complementary sequences, and primer extension give an exponential accumulation of the target fragments. The use of a thermostable DNA polymerase from *Thermus aquaticus* (Taq) has substantially improved the specificity, yield, sensitivity and length of targets that could be amplified, as well as simplifying the method and making it amenable to automation. The RT-PCR technique may be used to obtain either qualitative or quantitative determinations of cytokine gene expression.

In a qualitative RT-PCR determination of cytokine gene expression, it is only possible to indicate the presence or absence of the particular cytokine gene transcripts in the tissue sample by the detection of the RT-PCR products using the methods described in the next section. Qualitative RT-PCR is a commonly used technique in cytokine research to study acute allograft rejection in the different organ transplants cited in the previous section [Martinez et al, 1992; Krams et al, 1992; Whitehead et al, 1993].

The main drawback of qualitative RT-PCR is the inability to distinguish the difference in the level of cytokine gene expression. Moreover, the determination of positivity or negativity in a qualitative RT-PCR is dependent on the sensitivity of the detection technique used. Some form of quantitation of the cytokine gene expression signals is therefore desirable if changes in the level of expression of the cytokine

gene transcripts are to be demonstrated. Semi-quantitative RT-PCR where the level of gene expression of each cytokine is compared with the level of expression of a constitutionally expressed "housekeeping" gene like β-globin or actin as an internal standard, allows for the differential losses that occur during RNA recovery as well as standardising to some extent the subsequent reverse transcription and PCR. The popularity of the semi-quantitative RT-PCR technique is demonstrated by its widespread use in the study of acute allograft rejection [Dallman et al, 1991a; Lipman et al, 1992; Gaweco et al, 1995; Kirk et al, 1995; McLean et al, 1997].

The ultimate in quantitative RT-PCR is the use of competitive PCR, where a known amount of a synthetic mRNA (requiring simultaneous reverse transcription first) or cDNA construct is used as an internal standard and co-amplified in the same reaction tube with the cytokine gene transcripts to be quantitated using the same primers [Wang et al, 1989; Gilliland et al, 1990]. The competitor DNA fragment usually differs from the cDNA of interest by having either a small intron or a mutated restriction enzyme site, and the two PCR products can then be easily separated by gel electrophoresis after amplification. As the same PCR conditions are applied to both the competitor DNA fragment and the cDNA of interest, by titrating an unknown amount of the cDNA of interest against a dilution series containing known amounts of the competitor DNA fragment, the amount of the cDNA of interest can then be reliably quantitated. Theoretically, this technique provides a strategy for absolute and reproducible quantitation of cDNA by PCR. However, as the competitor is a DNA standard, there is no standardisation of the reverse transcription of the sample RNA into cDNA. Therefore, the use of a synthetic RNA construct as the competitor internal standard instead has been advocated to overcome this potential problem of differential efficiency in reverse transcription [Kanangat et al, 1992; Alms et al, 1996]. As quantitative RT-PCR is clearly a much more technically demanding technique, it is not surprising that there are only a few studies in transplantation cytokine literature that uses this technique [Strehlau et al, 1996; Strehlau et al, 1997].

1.7 Detection of RT-PCR products

It is obvious that just as the different RT-PCR methodologies described in the previous section have significant bearings on the type and quality of results obtained, it must be emphasized too that the method chosen for detecting and quantitating the amount of the resultant PCR products is equally important no matter which method of RT-PCR is chosen to amplify the gene transcripts of interest.

Several commonly used methods in the detection of RT-PCR products have been described in the current cytokine research literature. These methods are reviewed in the following paragraphs, not as an exhaustive treatise on the subject, but in order that the method chosen for the project may be put in its context. Although these methods are in common use in molecular biology laboratories, no reviews of the methods are available in the literature and so the references quoted in this section are only examples of the methods described.

The simplest method of detecting PCR products is the visual assessment of the "brightness" of bands on the image (captured on a Polaroid film, or more recently, captured digitally by a computer and subjected to manipulations before printing) produced after ultra-violet trans-illumination following electrophoresis of PCR products loaded in agarose or polyacrylamide gel with ethidium bromide staining of the PCR products [Martinez et al, 1992]. The presence of the appropriately sized PCR products can be predicted by the position of the primers on the cDNA sequence. The detection of small amounts of PCR products using this method is poor, and being a non-quantitative method, the differentiation of quantitative differences between the bands is crude. To enable a quantitative assessment of the "brightness" of the bands, the photographic image of the gel on a special film can be scanned by laser densitometry [Strehlau et al, 1997].

A more sensitive and specific method of detecting PCR products, which can also be used to quantify the amount of PCR products, involves the detection of radioactivity which had been incorporated into the PCR products. The PCR products are first

resolved by gel electrophoresis and the incorporated radioactivity may then be quantitated directly from the excised gel bands using a scintillation counter [Kanangat et al, 1992], or the gel can be dried on a special paper and the radioactivity analysed using a gel scanner [Kirk et al, 1995].

An alternative to the previous method is to transfer the PCR products onto a nylon membrane first by Southern blotting [Alms et al, 1996] or dot blotting [Dallman et al, 1991a], followed by radioactive-labelled oligonucleotide probing of the PCR products. The subsequent detection of the hybridised product/probe complex can be accomplished either by exposure on a radiographic film for qualitative analysis [Whitehead et al, 1993], or the radioactive signal can be quantitated directly by image scanning of the nylon membrane [O'Garra and Vieira, 1992]. An alternative to direct quantitation of the radioactive signal on the nylon membrane is the assessment of the intensity of the band on the autoradiograph using scanning densitometric analysis [Lipman et al, 1992].

A similar concept to the radioactive-based method described previously is using non-radioactive-labelled immunochemical oligonucleotide probes following the transfer of the PCR products onto a nylon membrane, with the exposure of the membrane-bound signal onto a radiographic film resulting from an enzyme-activated chemiluminescence. The signal detected on the radiographic film can be assessed qualitatively [Martinez et al, 1993], or the signal on the film can be quantitated using a laser densitometer [Wagner et al, 1997].

The latest method available at the commencement of this study was again a non-radioactive-based method called PCR ELISA. As the name suggest, it uses an enzyme-linked immuno-sorbent assay to detect the PCR products [Janezic et al, 1995]. This is a relatively novel method to detect PCR products with no published study in clinical transplantation demonstrating its use. This method was chosen for the project because it is highly sensitive in detecting PCR products (much more so than the gel electrophoresis method) and is relatively easy to use. Moreover, the spectrophotometric readings generated by the PCR ELISA provide a numerical

expression of the amounts of PCR products present which will allow semiquantitative comparison of level of gene expression at the different time points for each cytokine/CTL activation marker for the same patient. The details of this method will be described further in section 2.5 of chapter 2.

1.8 Outline of the project

This project aims to establish whether sequential monitoring of peripheral T cell cytokine gene expression can correlate and reflect the clinical immunological status of renal transplant patients.

In addition, the sequential changes in peripheral T cell cytokine gene expression may also shed light on their role in graft acceptance and acute rejection.

We set out to monitor the changes in the immunological status of patients following renal transplantation by studying the changes in peripheral T cell alloreactivity based on its cytokine and CTL activation marker gene expression profiles during the first six weeks following renal transplantation. There are several differences and unique features in our approach from the many studies reported in the literature and these are set out in the following paragraphs.

As many single time-point studies so prevalent in the cytokine literature can only provide a "snap-shot" of what is happening immunologically at the time of sampling, it is not surprising that conflicting findings about the association of certain cytokines with acute allograft rejection have been reported by these studies as discussed in the literature review set out in section 1.5. We have chosen to do a sequential study instead, so that the gene expression of the immune cells from each patient can be monitored longitudinally and the level of gene expression at the different sampling time points can be compared with one another.

Instead of monitoring the level of cytokine gene expression within the allografts as in most of the intragraft studies quoted in section 1.5, we chose to study the changes in the alloreactivity of peripheral blood T cells for several reasons. We have chosen to study T cells because they are the central player of the body's immune system orchestrating the complex allograft response seen during acute rejection. We accept that the population of T cells activated by the allograft would inevitably be diluted in the peripheral blood during its recirculation from the allograft back to the rest of the body, and therefore their contained immune activation signals would be faint in comparison to that obtained from within the graft itself. Nevertheless, as it is relatively easy to separate out the different cellular components of the circulating immune system, it would be possible to concentrate our study on the changes in alloreactivity of a single subset of immune cells, in this case the peripheral blood T cells. One could then attempt to correlate the gene expression profiles of peripheral T cells as a result of their exposure to the transplanted allograft over a period of time with the clinical course following renal transplantation, without the confounding problem of having multiple immune activation signals produced by the numerous cell populations contained in an intragraft sample. This problem is inevitable in intragraft studies since it is not possible to separate out the different immune and non-immune cell populations from needle core biopsies or fine needle aspirations obtained directly from the allografts, and this problem could also contribute to the many conflicting findings from such studies mentioned previously.

The validity of using peripheral T cells in our study to monitor intragraft alloreactivity has been supported by phenotypic studies which have shown that changes in the peripheral mononuclear cell subpopulations do reflect closely changes in these subpopulations within the allografts [Takahara et el, 1989; Tashiro et al, 1989]. Moreover, if immunological monitoring is to become a clinical reality in the future, then peripheral blood sampling as a means to achieve this would be a much more acceptable investigative modality to patients and clinicians alike than either needle core biopsy or fine needle aspirations.

The aim of our study was to investigate the changes in the alloreactivity of peripheral blood T cells following renal transplantation by monitoring the sequential changes in gene expression levels of Th1 and Th2 cytokines (i.e., to elucidate the nature of the Th1/Th2 paradigm in the periphery), as well as CTL activation markers, in peripheral blood samples during the early post renal transplant period, which we have limited to the first 6 weeks following renal transplantation. We were interested in establishing the cytokine/CTL activation marker gene expression profiles in the following groups of patients: namely, patients with no acute rejection episode (i.e. tolerant to the graft), patients with episode(s) of acute rejection responsive to anti-rejection therapies, and patients with acute rejection unresponsive to anti-rejection therapies.

A total of 6 cytokines and 2 CTL activation markers were studied in the project, and they were studied in two phases. In the first phase of the project, two Th1 cytokines (IL-2 and IFN-γ) and two Th2 cytokines (IL-4 and IL-10) were studied. Based on these preliminary results, we decided to study two other Th2 cytokines (IL-5 and IL-13) and two CTL activation markers (GrB and FasL).

Following full approval from the hospital's ethical committee for the project, patients were recruited from the renal transplant programme of the Wessex Renal and Transplant Unit based at St Mary's Hospital, Portsmouth. Peripheral blood samples were taken from recruited patients prior to transplantation and then at 4 time intervals following transplantation (day 2 to 3, day 5 to 7, day 10 to14 and around day 30) to cover the critical period when acute rejection commonly occurs. In addition, with each documented rejection episode, blood would be taken prior to the commencement of anti-rejection therapy, at the end of the anti-rejection therapy, and then at approximately 1 week and 1 month following anti-rejection therapy, so that the changes in peripheral T cell alloreactivity at the time of acute rejection and the effect of the treatment for the acute rejection episode could be monitored.

The cytokine/CTL activation marker gene expression of peripheral T cells were assayed indirectly using genetic engineering technology described in the previous section. In summary, the polymerase chain reaction technique was used to amplify

the cDNA derived from reverse transcription of the cytokine/CTL activation marker gene transcripts isolated from the peripheral blood T cells, and the resulting amount of specific amplified product was assayed using spectrophotometric quantitation following an enzyme-linked immunosorbent assay.

The T cell gene expression profile of each cytokine or CTL activation marker for each patient was obtained by measuring the gene expression of all the samples from each patient at the same time to enable suitable sequential semi-quantitative comparison in the level of gene expression between the different sampling time points. The details of the technique involved in isolating the T cells, the extraction, quantitation and standardisation of total RNA extracted from the T cells, the reverse transcription of the total RNA to cDNA, followed by the polymerase chain reaction and semi-quantitative detection of the amplified products are set out in the next chapter.

There are several potential benefits in monitoring changes in the immunological status of patients following renal transplantation which are clinically relevant. The most obvious benefit is that it may result in the earlier detection of acute rejection, thus facilitating an earlier and more effective treatment of the rejection episode, thereby reducing the degree of graft damage and consequently improving the long-term function of the allograft. The corollary of this benefit is the confident exclusion of the diagnosis of acute rejection as the cause of early graft dysfunction and allowing the diagnosis of cyclosporin A toxicity to be considered earlier and tested by a confident dose reduction.

Another potential benefit of immunological monitoring may be the achievement of one of the long term objectives in transplantation, that is, the maintenance of the allografts free of acute rejection episodes using the minimal amount of immunosuppressive drugs, both in terms of the dosages of the individual drug and the number of drugs used in the immunosuppressive regimens. This objective may be achievable if immunological monitoring can reliably detect a state of acquired graft tolerance thus allowing a safe and earlier reduction in immunosuppression in the post

transplant period, with the accompanying benefit of a reduction in the number and severity of opportunistic infections both in the short and medium term, and possibly of malignancies in the long term.

Finally, immunological monitoring may aid in the clinical decisions about continuing, changing or stopping the powerful immunosuppressive agents used to treat acute rejections. If immunological monitoring could predict the success or failure of an anti-rejection regime during the course of the treatment, it could enable an earlier switch to a different anti-rejection regimen, or even spare patients of the unavoidable associated morbidity of persisting with further immunosuppression, if immunological monitoring suggests a likely failure of the anti-rejection regimen used.

We hope that this project would contribute towards achieving some of these potential benefits of immunological monitoring of patients following their renal transplantation.

In summary, the specific issues addressed in this thesis are as follows:

1. the development of an efficient method of isolating T cells from peripheral blood.
2. the validation and further development of a semi-quantitative molecular assay of cytokine/CTL activation marker gene expression that is both reliable and reproducible.
3. the establishment of the sequential cytokine/CTL activation marker gene expression profiles in patients experiencing acute allograft rejection and those who do not.

Chapter 2: MATERIALS & METHODS

This chapter details all the materials and methods of all the experimental protocols used for the project and the statistical methods applied to the data collected. The first section deals with the cell separation protocol while the next 4 sections record all the molecular protocols, beginning with the main work with RNA (extraction of total RNA, its quantitation and standardisation), followed by reverse transcription, through the main polymerase chain reaction (PCR) protocols and finishing with the PCR ELISA (enzyme-linked immunosorbent assay) detection methodology. The penultimate section describes the verification of the integrity of the stored cDNAs by PCR of the house-keeping β-globin gene, and the final section gives a brief description of the statistical methodology used in the analysis of the data from the preliminary cell separation experiments and from the patients recruited in the project.

2.1 Cell separation

The first stage in the laboratory work-up for each peripheral blood sample taken from the patients was the separation of the peripheral blood lymphocytes from the rest of the blood components. The cell separation methodology evolved from detailed preliminary experiments on blood samples taken from healthy individuals as well as from post-transplant patients prior to the recruitment of patients for the study (see chapter 3 for details). The method finally chosen was selected to ensure the highest possible purity of lymphocytes obtainable while avoiding prolonged and excessive in vitro manipulation of the cells which has the potential of influencing the gene expression signal by causing not only the induction of a particular cytokine mRNA, but also the decline in the level of unstable cytokine mRNA [Vossen and Savelkoul, 1994].

Principle:

Cell separation

The mononuclear cells from the peripheral blood were first isolated by density gradient centrifugation using Lymphoprep. Following the centrifugation, the peripheral blood sample would be separated into four layers based on the density of the various blood components relative to the Lymphoprep, namely (from the bottom of the tube): (1) the red blood cells clumped together with the granulocytes; (2) a clear Lymphoprep layer; (3) a narrow and variable-sized band of "buffy coat" containing the mononuclear component of the white blood cells; (4) the straw-coloured plasma finally as the top layer. The "buffy coat" layer of mononuclear cells was carefully retrieved and subjected to the next step of cell purification.

Cell purification

The aim of this step is to produce a highly enriched preparation of T lymphocytes. This consisted of a sequential combination of non-specific and specific methods to remove monocytes, B cells and NK cells. The non-specific physical method of adherence onto plastics by monocytes was based on their phagocytic properties [Koller et al, 1973]. Platelets were also removed during this step because of their inherent adherent properties upon activation. The specific method, termed panning, involved using mouse anti-human monoclonal antibodies to remove the remaining monocytes, B lymphocytes and NK cells by negative selection onto plates coated with rabbit anti-mouse antibodies [Wysocki and Sato, 1978]. At the end of the panning step, the remaining mononuclear cells consist essentially of enriched T lymphocytes.

Consumables:

1. Lymphoprep, specific gravity 1.077 g/l [Nycomed, UK]
2. Phosphate buffered saline (PBS) **without** calcium or magnesium (10X) [Life Technologies, UK]
3. Dulbecco's PBS (**with** calcium and magnesium) (10X) [Life Technologies, UK]
4. RPMI 1640 (with 25 mM HEPES & L-glutamine) [Life Technologies, UK]

5. Sodium pyruvate (100 mM) [Life Technologies, UK], 1 ml added per 100 ml RPMI 1640
6. L-glutamine (200 mM) [Life Technologies, UK], 1 ml added per 100 ml RPMI 1640
7. Fetal calf serum (FCS) [Life Technologies, UK], heat inactivation of complements at 56 ^{0}C for 30 mins and filtered prior to use
8. Rabbit anti-mouse antibodies (1.6 g/l) [Dako, UK]
9. Monoclonal mouse anti-human antibodies to CD 11b (MCA551X), CD 14 (MCA596XZ), CD 16 (MCA1193XZ) & CD 19 (MCA662X) - all at 1 mg/ml [Serotec, UK]
10. Sigma Trizma Base (Sigma, UK) - 0.05 M Tris (hydroxymethyl)aminomethane, pH 9.5 prepared by dissolving 3.03 g of Trizma base in 500 ml of sterile distilled water, adjusting the pH to 9.5 with 2.5 M hydrochloric acid
11. Sterile 50 ml skirted conical-based polypropylene tubes [Greiner Labortechnik, UK]
12. Sterile 15 ml conical-based polypropylene tubes [Greiner Labortechnik, UK]
13. Tissue culture grade petri dishes [Greiner Labortechnik, UK]
14. Sterile 3.5 ml Pasteur pipettes [Greiner Labortechnik, UK]
15. Sterile petri dishes [Bibby Sterilin, UK]
16. 10 ml Becton Dickinson Vacutainers tubes with sodium citrate [Hospital Management & Supplies, UK]
17. 0.22 μ filters [Life Sciences, UK]

Equipment:

1. MSE Mistral 3000i centrifuge [Sanyo, UK]
2. Stuart Scientific tilting tube roller with 5 rollers providing rocking and rolling shaking [Philip Harris Scientific, UK]
3. Water-jacket incubator, Model 3157 [Forma Scientific, USA]
4. Techne MWB-10L microcarrier water bath [Techne (Cambridge), UK]
5. Microflow pathfinder laminar flow cabinet [MDH, UK]

Method:

For each sampling, up to 20 ml of peripheral blood was collected into two 10 ml vacutainer tubes, each tube containing 1 ml of sodium citrate as anti-coagulant. The volume of blood collected was variable because of the nature of the tubes used (variability in the amount of vacuum within the tubes) and dependent on the duration that each tube was connected to the vacutainer collection system during venesection.

The volume of blood for cell separation was first diluted 1 in 1 with PBS/2% FCS in a sterile 50 ml polypropylene tube. About 10 ml of the diluted blood was then carefully layered onto 4 ml of Lymphoprep in a sterile 15 ml conical-based polypropylene tube. Usually three or four of these tubes were used for each patient's blood sample. The tubes were spun at 2500 rpm (1100g) for 20 mins in an MSE Mistral 3000i centrifuge at room temperature (set at 20 ^{0}C) and without any acceleration or brake settings.

Following centrifugation, the "buffy coat" containing the mononuclear cells was carefully removed onto a sterile 50 ml polypropylene tube using a Pasteur pipette. The mononuclear cells were then washed once with PBS/2% FCS and the cells pelleted by centrifuging at 1500 rpm (400g) for 10 mins with maximum acceleration and brake applied.

The pelleted cells were resuspended in 8 ml of warm tissue culture medium (RPMI 1640 with HEPES, L-glutamine, sodium pyruvate and 10% FCS). The cell suspension was then incubated on 2 tissue culture grade petri dishes in an incubator at 37 ^{0}C for 30 mins. The non-adherent cells were removed onto a sterile 50 ml polypropylene tube by gently washing the petri dishes with warm tissue culture medium using the Pasteur pipette. The cells were again pelleted by centrifuging at 1500 rpm (400g) for 10 mins with maximum acceleration and brake applied.

Table 2.1.1 – Monoclonal antibodies used for panning and the cells they identify

mAb	**Mononuclear cells identified by the monoclonal antibodies**		
CD11b	Monocytes	B cells	NK cells
CD14	Monocytes	-	-
CD16	Monocytes	-	NK cells
CD19	-	B cells	-

The pelleted cells were resuspended in 400 µl of Dulbecco's PBS/5% FCS and 10 µl of each of the 4 monoclonal antibodies (to CD 11b, 14, 16 & 19) were added to the cell suspension. The different mononuclear cells which these monoclonal antibodies identify is listed in table 2.1.1. The cell suspension was then incubated at 4 ^{0}C (by placing the 50 ml polypropylene tube containing the cell suspension in a bottle filled with ice) on a Stuart roller for 30 mins to ensure thorough mixing.

The excess antibodies were then washed off with Dulbecco's PBS/5% FCS and centrifuging at 1500 rpm (400g) for 10 mins with maximum acceleration and brake applied. The pelleted cells were resuspended in 8 ml of Dulbecco's PBS/5% FCS and incubated on a Sterilin petri dish coated with rabbit anti-mouse antibodies for 45 mins at 4 ^{0}C (by placing the plate in the fridge). Each Sterilin petri dish was prepared by incubating 50 µl of rabbit anti-mouse antibodies (at 1.6 g/l) in 8 ml of 0.05 M Tris (at least overnight incubation at 4 ^{0}C) and then blocked against non-specific adherence with Dulbecco's PBS/2% FCS for at least 1 hour prior to use.

The non-adherent cells were recovered onto a sterile 50 ml polypropylene tube by gentle washing of the Sterilin petri dish with Dulbecco's PBS/5% FCS using a Pasteur pipette, reconstituting the volume to 15 ml final volume. 2 ml of the cell suspension was removed for cell counting, while the remaining cells were pelleted by centrifuging at 1500 rpm (400g) for 10 mins with maximum acceleration and brake

applied. The cell count was performed using a Coulter counter by the Haematology department at St Mary's Hospital.

2.2 Extraction of total RNA , RNA quantitation & standardisation

Extraction of total RNA from the cell pellet was commenced by homogenising the cell pellet in 1 ml of RNAzol B, a commercial preparation for undegraded RNA of high purity. The homogenate was transferred to a sterile 1.5 ml eppendorf for temporary storage in a minus 20 ^{0}C freezer.

The extraction of total RNA from the cell pellet was based on the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction [Chomczynski and Sacchi, 1987]. This method provided a high yield of undegraded RNA preparations with high purity.

Consumables: all the reagents and pipette tips were strictly allocated for RNA use only to prevent inadvertent contamination with ribonucleases.

1. RNAzol B [Biogenesis, UK], a commercial preparation containing guanidinium thiocyanate, phenol and β-mercaptoethanol. Guanidinium thiocyanate, a potent denaturing agent [Cox, 1968], together with the reducing agent β-mercaptoethanol [Sela et al, 1957], are both potent inactivators of ribonucleases.
2. 0.1% diethyl pyrocarbonate (DEPC) [Sigma, UK] in distilled water (DEPC-water) – this is a strong, but not absolute, inhibitor of ribonucleases [Fedorcsak and Ehrenberg, 1966] and is prepared by adding 100 μl of DEPC to 100 ml of double-distilled water, leaving the solution overnight in a fume cupboard and then autoclaving the solution.
3. Chloroform [Merck, UK]
4. Isopropanol [Merck, UK]
5. 75% ethanol - prepared by dilution of absolute ethanol [James Burroughs, UK] with DEPC-water

6. Sterile pipette tips of various sizes [Greiner Labortechnik, UK]
7. Sterile 1.5 ml eppendorfs [Greiner Labortechnik, UK]

Equipment:

1. Heraeus Sepatec Contifuge 17RS centrifuge [Heraeus, UK]
2. Windsor Incubator, set at 45 ^{0}C [Sandrest, UK]
3. Whirlimixer (vortex) [Jencons Scientific, UK]
4. Water bath, set at 55 ^{0}C [Grant Instruments, UK]
5. GeneQuant RNA/DNA Calculator [Pharmacia, UK]
6. Ultramicrovolume cell (5 µl) [Pharmacia, UK]
7. Pipettes of various volume ranges (Gilson, Eppendorf, Biohit) [Anachem, UK; Merck, UK; Alpha Laboratories, UK]

Method: RNA extraction

The RNAzol B homogenates were completely thawed out during their transport from Portsmouth to the Molecular Biology Laboratory in Southampton. On arrival, they were placed on ice to reduce the risk of RNA degradation.

The centrifuge was first cooled down to 4 ^{0}C prior to use. 100 µl of chloroform was added to each eppendorf of RNAzol B homogenate. The eppendorf was shaken (rather than vortexed) vigorously for at least 15 sec and then left to stand on ice for 5 mins. The mixture was then centrifuged at 12000 rpm (12000g) for 15 mins at 4 ^{0}C.

Following centrifugation, the homogenate separated into two phases, a lower bluish phenol-chloroform phase and a colourless upper aqueous phase containing the RNA, with a whitish interphase containing DNA and proteins. The upper aqueous phase (usually 500 to 600 µl) was transferred into a sterile 1.5 ml capped eppendorf, carefully avoiding the interphase.

The RNA was precipitated in isopropanol. 600 µl of isopropanol was added to the aqueous phase, the two solutions were thoroughly mixed by the vortexing. The

mixture was left to stand on ice for about 45 mins to allow the RNA to precipitate. The precipitated RNA was then pelletted by centrifuging at 12000 rpm (12000g) for 15 mins at 4 ^{0}C. The supernatant was discarded, carefully avoiding the RNA pellet which appeared as a whitish streak at the bottom of the eppendorf.

The RNA pellet was washed by vortexing in 500 µl of cold 75% ethanol (stored at minus 20 ^{0}C) before being pelleted again by centrifuging at 7500 rpm (7500g) for 8 mins at 4 ^{0}C. The ethanol was removed to dryness, again carefully avoiding the RNA pellet, and the RNA pellet was then dried by leaving the eppendorf open in a sterile petri dish placed in an incubator (set at 45 ^{0}C) for about 10 to 15 mins. It was important to avoid over-drying the RNA pellet as it would make its solubilization difficult.

Depending on the size of the RNA pellet, between 15 and 50 µl of DEPC-water was added to solubilize the RNA pellet. To aid solubilization, the eppendorf containing the RNA solution was placed in a 55 ^{0}C water bath for 5 mins prior to quantitation. The eppendorf containing the RNA solutions were placed on ice upon removal from the 55 ^{0}C water bath.

Method: RNA quantitation

The setup of the GeneQuant RNA/DNA calculator was first set for RNA quantitation by the following criteria:

- path length = 5
- use 320 nm? = Yes
- RNA factor = 40.0
- bases number (A, C, G, T, U) = 0
- oligo length = 1
- mw calc = 0.0
- ratio expected = 2.000
- conc. expected = 0.000
- protein coeff. 1 = 1.550; protein coeff. 2 = 0.760

Prior to using the ultramicrovolume cell for each reading, the cell was cleaned by sequentially flushing the cell chamber with 0.1 M sodium hydroxide, 0.1M hydrochloric acid, ultra-high quality water and DEPC-water, flicking the fluid off each time. The cell was wiped dry with tissue paper and the cell chamber aspirated to dryness using a Cristal-tip on a 10 µl pipette.

The GeneQuant was zeroed by using DEPC-water as the reference. For each quantitation, 5 µl of the neat RNA solution or its appropriate dilution was placed in the cleaned cell chamber. The absorbance at 260 nm and 280 nm, its ratio and the RNA concentration in µg/ml was read from the GeneQuant. A 260/280 ratio of greater than 1.9 was expected from the RNA extraction technique used.

When the absorbance at 260 nm was high (eg. nearly or greater than 3.000) resulting in a 260/280 ratio of less than 1.9, a dilution of the RNA solution was quantitated and the original RNA concentration calculated from the readings made of the dilution.

Method: RNA standardisation

Once the diluted RNA solutions were quantitated, the concentrations of the undiluted RNA solutions were calculated by multiplying the concentrations of the RNA solutions by the dilution factor of the RNA solutions used in the quantitation.

The RNA solutions were standardised into 1 µg of total RNA in 8 µl volumes prior to reverse transcription by diluting the calculated amount of RNA solution with the corresponding volume of DEPC-water.

Following some preliminary experiments, it was found that 1 µg of total RNA would be an appropriate quantity of RNA for reverse transcription both in terms of the corresponding quantity of first-strand cDNA necessary for successful cytokine PCR and the available quantity of total RNA extractable from the often lymphopaenic post-transplant patients.

Four 1 μg aliquots of RNA solutions (8 μl each) for each patient sample were prepared whenever possible. To simplify the reverse transcription procedure, all the 1 μg aliquots of RNA were combined into a single 0.75 ml eppendorf tube for the denaturation step before aliquoting the 1 μg of RNA solution into the cDNA synthesis reaction tubes. To compensate for evaporative loss during the denaturation step, about 1 to 2 μl of extra DEPC-water were added to the combined aliquots of RNA solutions (depending on the amount of cDNA to be synthesized) during the standardisation procedure.

2.3 First-strand cDNA synthesis (reverse transcription)

The reverse transcription of total RNA extracted was catalyzed by Moloney Murine Leukaemia Virus (M-MuLV) reverse transcriptase. The First-Strand cDNA Synthesis Kit from Pharmacia Biotech provide all the reagents necessary to generate full-length first-strand cDNAs from RNA templates 7 kilobases or more in length using primers provided.

Consumables: Kit components used for the reverse transcription

1. Bulk first-strand cDNA reaction mixes - cloned, FLP *Cpure*® Murine Reverse Transcriptase, RNAguard, RNase/DNase-Free BSA, dATP, dCTP, dGTP and dTTP in aqueous buffer.
2. DTT solution - 200 mM aqueous solution of dithiothreitol (a reducing agent).
3. *Not* I-d$(T)_{18}$ bifunctional primer - an aqueous solution at 5 μg/μl of RNA (primer sequence: 5'-d[AACTGGAAGAATTCGCGGCCGCAGGAAT_{18}]-3'), diluted 1 in 25 prior to use.
4. RNase-free water - treated with diethyl pyrocarbonate (DEPC).

Equipment:

1. Amplirad UV cabinet [Gene Research Instrumentation, UK] - previously sterilised 0.75 ml eppendorf tubes were UV-irradiated for at least 10 minutes before use.

2. Laminar airflow cabinet [MDH, UK]
3. Pipettes of various volume ranges (Gilson, Eppendorf) [Anachem, UK; Merck, UK]
4. Hybaid thermal reactor [Hybaid, UK]
5. Whirlimixer (vortex) [Jencons Scientific, UK]
6. Water bath, set at 37 ^{0}C [Grant Instruments, UK]

Method:

Following standardisation of the total RNA into 1 μg (in 8 μl volume) aliquots, the standardised RNA solutions were heated to 65 ^{0}C for 10 minutes using a Hybaid thermal reactor to denature the RNA before being chilled in ice for 5 to 10 mins. While the RNA solutions were being heated, cDNA reaction tubes were set up by adding 5 μl of bulk first-strand reaction mix, 1 μl of DTT and 1 μl of *Not* I-d$(T)_{18}$ primer into labelled 0.75 ml sterile eppendorf tubes (UV-irradiated).

Next, 8 μl of the denatured RNA solutions were transferred to each cDNA reaction tube (making a total volume of 15 μl each) and the resulting reaction mixture was mixed thoroughly by pipetting it in and out. The cDNA reaction tubes were incubated in a 37 ^{0}C water bath for one hour before being stored at minus 70 ^{0}C for subsequent molecular analysis.

2.4 Polymerase chain reaction (PCR) for cytokine and cytotoxic T-lymphocyte (CTL) activation marker gene expression

The PCR protocols used in the project were modified from the protocols developed by the Molecular Immunology Laboratory of the Department of Medicine, University of Southampton. The cytokine primer sequences and their biotinylated capture probe sequences used in the PCR ELISA detection system were also kindly provided by the Department of Medicine.

As for the CTL activation markers, the primer sequences for granzyme B and fas ligand were obtained from a recent paper by Strom's group [Strehlau et al, 1997]. The capture probe sequences for use in the PCR ELISA system for both PCR products were kindly designed for us by John Holloway (Molecular Genetics Group, University of Southampton), using the OLIGO 5 programme.

The preliminary experimental work on all the molecular protocols used in the project is detailed in chapter 4.

Consumables:

1. Ultra-violet light irradiated double-distilled water (ddH_2O)
2. Magnesium chloride ($MgCl_2$) at 25 mM concentration [Boehringer Mannheim, UK]
3. PCR reaction buffer **without** magnesium chloride, 10X concentration, containing 100 mM Tris-HCl, 500 mM KCl, pH 8.3 [Boehringer Mannheim, UK]
4. PCR digoxigenin (DIG)-labelling mix, containing 2 mM dATP, dCTP, dGTP each, 1.9 mM dTTP, 0.1 mM digoxigenin-11-dUTP, pH 7.0 [Boehringer Mannheim, UK]
5. Cytokine and CTL activation marker primers, diluted from stock solutions to 10 μM concentrations [R & D Systems, UK & Eurogentec, UK]
6. *Thermus aquaticus* (*Taq*) polymerase at 5 U/μl [Boehringer Mannheim, UK]
7. 1.5 ml eppendorfs [Greiner Labortechnik, UK]

8. 0.2 ml thin-walled PCR tubes/caps (strip of 8 tubes/caps each) [Advanced Biotechnologies, UK]
9. Sterile pipette tips of various sizes [Greiner Labortechnik, UK]

Oligonucleotide primer sequences (5' to 3'):

1) Interleukin-2 (IL-2): product size 255 base pairs (as amplified from cDNA)

Sense primer - GCC ACA GAA CTG AAA CAT CT

Anti-sense primer - AGT CAG TGT TGA GAT GAT GC

2) IL-4: product size 449 base pairs (as amplified from cDNA)

Sense primer - CTG CAA ATC GAC ACC TAT TA

Anti-sense primer - GAT CGT CTT TAG CCT TCC

3) Interferon gamma (IFN-γ): product size 270 base pairs (as amplified from cDNA)

Sense primer - GGT CAT TCA GAT GTA GCG GA

Anti-sense primer - GCG TTG GAC ATT CAA GTC AG

4) IL-10: product size 231 base pairs (as amplified from cDNA)

Sense primer - CTT GTC TGA GAT GAT CCA G

Anti-sense primer - CTC ATG GCT TTG TAG ATG CC

5) IL-5: product size 257 base pairs (as amplified from cDNA)

Sense primer - CTG AGG ATT CCT GTT CCT GT

Anti-sense primer - CAA CTT TCT ATT ATC CAC TC

6) IL-13: product size 500 base pairs (as amplified from cDNA)

Sense primer - CGG TCA TTG CTC TCA CTT GCC TT

Anti-sense primer - TTA CCC CTC CCT AAC CCT CCT T

7) Granzyme B (GrB): product size 431 base pairs (as amplified from cDNA)

Sense primer - GGG GAA GCT CCA TAA ATG TCA CCT

Anti-sense primer - TAC ACA CAA GAG GGC CTC CAG AGT

8) Fas ligand (FasL): product size 301 base pairs (as amplified from cDNA)

Sense primer - GCC TGT GTC TCC TTG TGA

Anti-sense primer - GCC ACC CTT CTT ATA CTT

Equipment:

1. Elgastat Option 4 water purifier (for double-distilled water) [Elgaserve, UK]
2. Amplirad ultra-violet light cabinet [Genetic Research Instrumentation, UK]
3. Laminar airflow cabinet [MDH, UK]
4. Whirlimixer (vortex) [Jencons Scientific, UK]
5. Pipettes of various volume ranges (Gilson, Eppendorf, Biohit) [Anachem UK; Merck, UK; Alpha Laboratory, UK]
6. Perkin-Elmer GeneAmp System 9600 thermal cycler [Perkin-Elmer, UK]

Method:

Prior to the start of PCR experiments, sterile 1.5 ml eppendorfs and double-distilled water were first irradiated with UV light for 10 to 15 mins to destroy any inadvertent contamination of the experiments with foreign DNA. The cDNA from each patient series and the reagents for PCR were thawed out in ice.

A master reagent mix was made for each PCR by using the volumes (in μl) of each reagent as set out in table 2.4.1 for IL-2, IL-13 and IFN-γ, table 2.4.2 for IL-4 and IL-10 and table 2.4.3 for IL-5, GrB and FasL, depending upon the number of samples in each patient series to be analysed per PCR run.

The total volume of master reagent mix for each patient sample series set out in the tables include an extra aliquot for the negative control (using UV-irradiated double-distilled water) for each PCR. Moreover, to overcome the inherent inaccuracies (small but nevertheless unavoidable) arising from multi-aliquot pipetting, and to ensure that the sufficient master reagent mix was available for each sample, an excess aliquot of each PCR master reagent mix was prepared. Each reagent was vortexed immediately prior to aliquoting into the master reagent mix.

Table 2.4.1 - Master reagent mix for IL-2, IL-13 and IFN-γ PCR

Number of samples in patient series	**5**	**6**	**7**	**8**
ddH_2O - 12.5 μl*	87.5	100.0	112.5	125.0
$MgCl_2$ (25 mM) - 2.0 μl*	14.0	16.0	18.0	20.0
Boehringer 10X reaction buffer (without $MgCl_2$) - 2.5 μl*	17.5	20.0	22.5	25.0
DIG-labelling mix - 2.5 μl*	17.5	20.0	22.5	25.0
Sense primer (10 μM) - 1.5 μl*	10.5	12.0	13.5	15.0
Anti-sense primer (10 μM) - 1.5 μl*	10.5	12.0	13.5	15.0
Taq* polymerase (Boehringer) - 0.2 μl	1.4	1.6	1.8	2.0

Notes: * volume of reagent per sample; all the volumes are in μl

Table 2.4.2 - Master reagent mix for IL-4 and IL-10 PCR

Number of samples in patient series	**5**	**6**	**7**	**8**
ddH_2O - 12.0 μl*	84.0	96.0	108.0	120.0
$MgCl_2$ (25 mM) - 2.5 μl*	17.5	20.0	22.5	25.0
Boehringer 10X reaction buffer (without $MgCl_2$) - 2.5 μl*	17.5	20.0	22.5	25.0
DIG-labelling mix - 2.5 μl*	17.5	20.0	22.5	25.0
Sense primer (10 μM) - 1.5 μl*	10.5	12.0	13.5	15.0
Anti-sense primer (10 μM) - 1.5 μl*	10.5	12.0	13.5	15.0
Taq* polymerase (Boehringer) - 0.2 μl	1.4	1.6	1.8	2.0

Notes: * volume of reagent per sample; all the volumes are in μl

Table 2.4.3 - Master reagent mix for IL-5, GrB & FasL PCR

Number of samples in patient series	**5**	**6**	**7**	**8**
ddH_2O - 13.5 μl*	94.5	108.0	121.5	135.0
$MgCl_2$ (25 mM) - 1.0 μl*	7.0	8.0	9.0	10.0
Boehringer 10X reaction buffer (without $MgCl_2$) - 2.5 μl*	17.5	20.0	22.5	25.0
DIG-labelling mix - 2.5 μl*	17.5	20.0	22.5	25.0
Sense primer (10 μM) - 1.5 μl*	10.5	12.0	13.5	15.0
Anti-sense primer (10 μM) - 1.5 μl*	10.5	12.0	13.5	15.0
Taq* polymerase (Boehringer) - 0.2 μl	1.4	1.6	1.8	2.0

Notes: * volume of reagent per sample; all the volumes are in μl

2.5 μl of ddH_2O (serving as negative control) and 2.5 μl of each cDNA in the patient sample series (thoroughly mixed first by pipetting in and out several times) were aliquoted into previously labelled 0.2 ml PCR tubes, the number of tubes per strip being adjusted according to the number of samples in each patient sample series. The *Taq* polymerase (kept at minus 20 ^{0}C) was added to the master reagent mix immediately before it was aliquoted into the PCR reaction tubes containing the cDNA or ddH_2O. 22.5 μl of each PCR reagent mix (thoroughly mixed by vortexing prior to use) was added to each PCR tube containing ddH_2O or cDNA, making a total PCR reaction volume of 25 μl.

The PCRs were performed in the Perkin-Elmer 9600 thermal cycler using the program set out in table 2.4.4 and their corresponding optimal annealing temperature (Ta) in table 2.4.5. The annealing temperature for each primer pairs was deduced experimentally by the Molecular Immunology Laboratory of the Department of Medicine, University of Southampton, for all the cytokine primers while the annealing temperature for the two CTL activation markers were obtained from a published source [Strehlau et al, 1997]. Although a single annealing temperature and

PCR condition was used for all 15 different PCRs in that paper, my preliminary experiments have pointed to a higher optimal annealing temperature of 60 ^{0}C for granzyme B while the published optimal annealing temperature stated for fas ligand was found to be satisfactory.

Table 2.4.4 - Cytokine PCR program

• Denaturation: 95 ^{0}C for 1 minute
• 3 temperature PCR cycle with the following profile for 35 cycles: 94 ^{0}C for 20 seconds, ramp over 45 seconds, optimum Ta for 30 seconds, ramp over 45 seconds, 72 ^{0}C for 1 minute
• Final extension: 72 ^{0}C for 10 minutes
• Hold at 10 ^{0}C until the PCR products were stored at 4 ^{0}C

Table 2.4.5 - Optimal annealing temperatures (Ta)

Cytokine/CTL activation markers	**Ta (^{0}C)**
IL-2 and IFN-γ	54
IL-4 and IL-5	50
IL-10	52
IL-13 and GrB	60
FasL	55

The PCRs were conducted in two batches. IL-2, IL-4, IL-10 and IFN-γ PCRs were performed in the first batch and IL-5, IL-13, GrB and FasL were performed in the second. To save time, the PCR in each batch with the same annealing temperature were performed together. Hence, in the first batch, IL-2 and IFN-γ PCRs were performed together while IL-4 and IL-10 PCRs were performed separately. For the second batch, IL-13 and GrB PCRs were performed at the same time, while IL-5 and

FasL PCRs were performed separately. Each PCR run took approximately two and a half hours to complete. For each batch, all the four PCRs in the batch for each patient sample series were performed on the same day. The PCR products were stored at 4 ^{0}C for the ELISA detection of the PCR products as soon as possible (all within 4 days).

2.5 Detection of cytokine and CTL activation marker PCR products

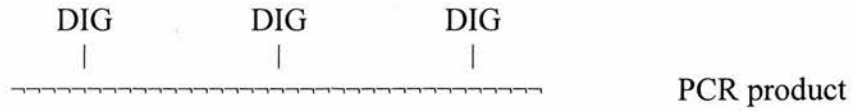
Principle of PCR ELISA

This is an extremely sensitive method to detect PCR products, being about a thousand-fold more sensitive than the resolution obtainable using gel electrophoresis (Janezic et al, 1995). The basic principle of the method is illustrated in figure 2.5.1.

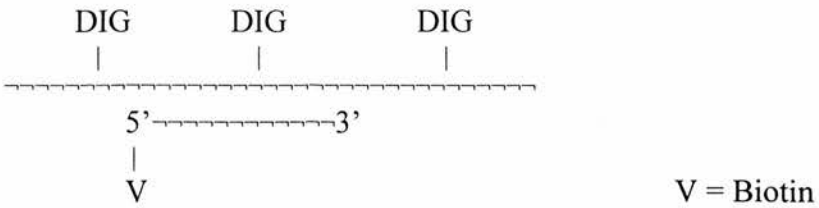
The first stage involves the generation of PCR products containing digoxigenin which was described in the previous section on cytokine PCR. The digoxigenin-labelled PCR products are then detected by cytokine-specific biotinylated capture probes which hybridise with an internal sequence of the digoxigenin-labelled PCR products. The digoxigenin-labelled PCR product/ cytokine-specific biotinylated capture probe "dimers" are firmly immobilised onto streptavidin coated wells by the interaction between the biotin on the capture probes and the streptavidin coated at the bottom/side of each well. Repeated washing of the wells following a period of incubation result in only the specific cytokine PCR product/capture probe dimers being retained in the wells. The digoxigenin moieties in the PCR products are then detected using an anti-digoxigenin (anti-DIG) antibody conjugated to a peroxidase (POD) enzyme. The peroxidase enzyme reacts with a substrate to give a colour change which is quantitated spectrophotometrically (figure 2.5.2).

Figure 2.5.1 - Principle of PCR ELISA

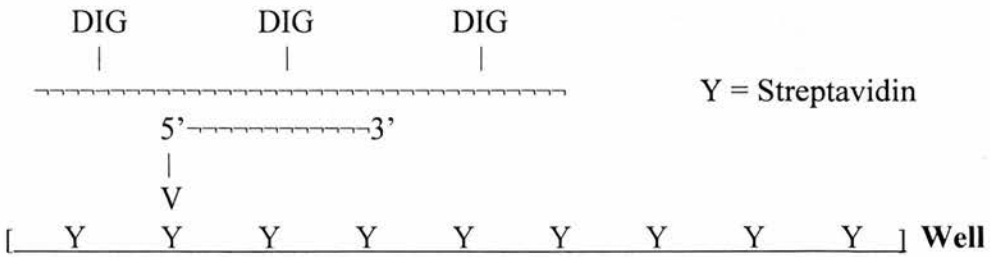
1. PCR - using DIG-labelling mix for the PCR (DIG-11-dUTP 0.01mM, 0.19 mM dTTP and 0.2 mM of dATP, dCTP & dGTP)
   - serial dilutions of DIG-labelled PCR products which were denatured by NaOH



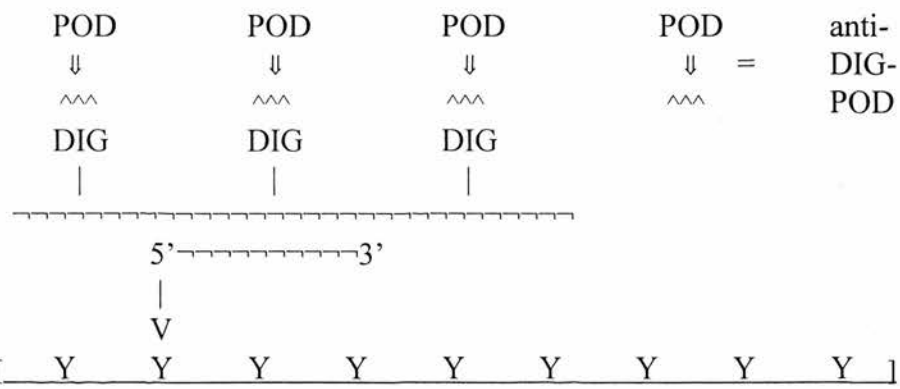
2. Capture probe (20 bp) - hybridises with internal part of PCR product (3 hours incubation on shaker at 37 ^{0}C)



3. Biotin on 5' end of capture probe immobilised onto streptavidin coated well



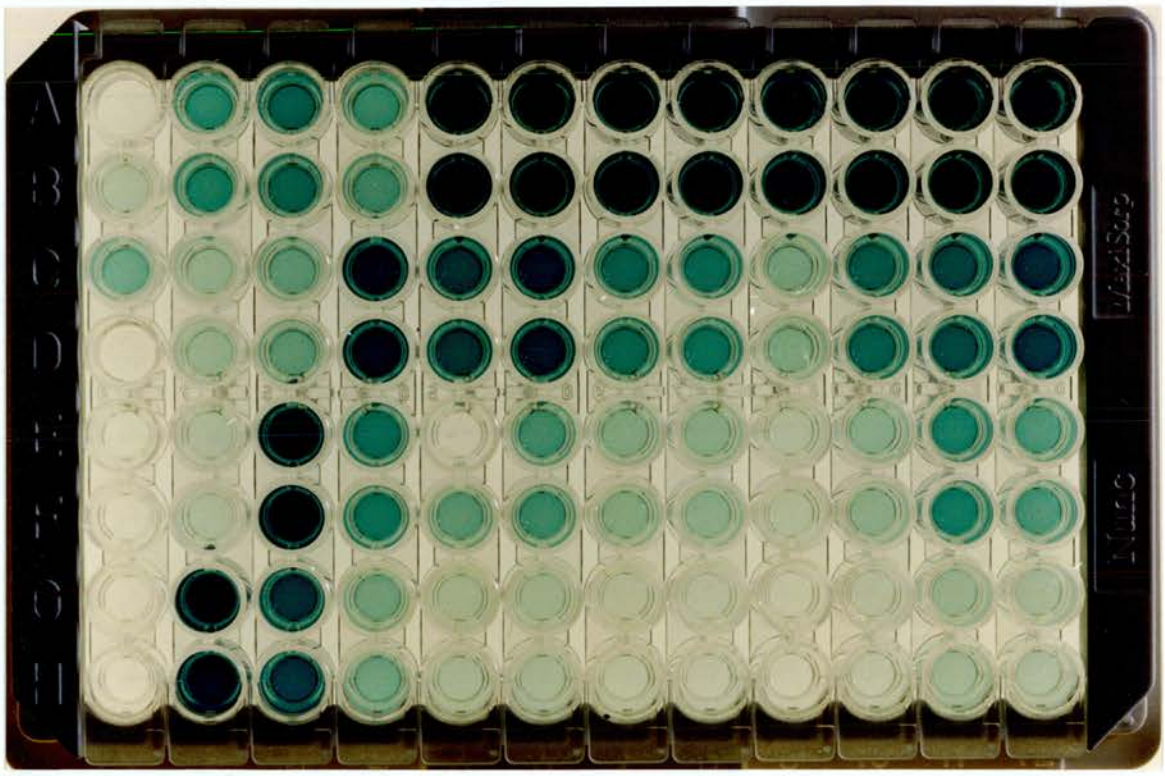
4. DIG-detection using an antibody-enzyme peroxidase system - peroxidase-conjugated anti-digoxigenin antibody (anti-DIG-POD)



5. PCR product visualised by colour change (green) with addition of enzyme substrate (ABTS).

6. Spectrophotometric quantitation by colour absorbance at 405 nm by ELISA plate reader.

Figure 2.5.2 – An ELISA plate ready for reading – the intensity of the colour change in each pair of duplicate wells is dependent on the amount of PCR products present.



As the ELISA detection of PCR products from the batch of PCR runs for each patient sample series were performed on the same ELISA plate (i.e. PCR products from the same time point for each cytokine/CTL marker in each batch of PCRs were analysed on the same plate), the quantitative differences in the amount of PCR product levels detectable using the PCR ELISA allows the differences in the level of gene expression between the different samples of each patient sample series to be compared in a semi-quantitative way for each cytokine or CTL activation marker.

Consumables:

1. Sterile 1.5 ml and 0.75 ml eppendorfs [Greiner Labortechnik, UK]
2. Sterile pipette tips of various sizes [Greiner Labortechnik, UK]
3. Eppendorf Combitips 0.5 ml & 5 ml [Merck, UK]
4. 20 ml universal containers [Bibby Sterilin, UK]

5. 50 ml polypropylene tubes [Greiner Labortechnik, UK]
6. Cytokine biotinylated capture probes, diluted to 5 ng/µl [R & D Systems, UK]
7. PCR ELISA kit as detailed below [Boehringer Mannheim, UK]
8. Double-distilled water (ddH_2O)
9. Aluminium foils [Merck, UK]

PCR ELISA kit components:

1. Washing solution - this was prepared by dissolving one washing tablet (provided in the kit) in 2 l of double-distilled water. The solution was stable for 6 weeks at 4 ^{0}C.
2. Anti-digoxigenin-peroxidase conjugate (anti-DIG-POD) - 250 mU as stabilized lyophilisate, resuspended in 250 µl of double-distilled water, mixing carefully for 15 mins to ensure full reconstitution. Reconstituted conjugate was stable for about 2 months at 4 ^{0}C
3. Denaturation solution containing sodium hydroxide (NaOH)
4. Conjugate dilution buffer
5. Hybridisation buffer
6. Substrate buffer
7. Substrate tablets - 5 mg ABTS® (2,2-Azinodi-3-ethylbenzthiazoline sulphonate [6]) per tablet
8. Microtitre plate (MTP) modules (8 wells per module) - the wells were precoated with streptavidin and postcoated with blocking agent
9. Strip frame for 8-well MTP modules (maximum of 12 strips per frame)
10. Cover foils for MTP

Biotinylated capture probe sequences (5' to 3'): biotin at 5' end

1) IL-2 - CTG AAC AGA TGG ATT ACC TT
2) IL-4 - CCG TAA CAG ACA TCT TTG CT
3) IL-10 - TGA GAA CCA AGA CCC AGA CA
4) IFN-γ - CAC TCT TTT GGA TGC TCT GG
5) IL-5 - GGG AAT AGG CAC ACT GGA GA
6) IL-13 - CGA GAA GAC CCA GAG GAT GC

7) GB - AGG AAC AGG AGC CGA CCC AG

8) FasL - TAG GCC ACC CCA GTC CAC CC

Equipments:

1. Pipettes of various sizes (Gilsons, Eppendorfs) [Anachem, UK; Merck, UK]
2. Eppendorf Multipette 4780 [Merck, UK]
3. Biohit Proline 500 electronic pipette [Alpha Laboratories, UK]
4. Finnepipette 8-channel electronic multiple-pipette [Life Sciences, UK]
5. Jencons Mixamatic (Vortex) [Jencons Scientific, UK]
6. DPC Micromix 5 microtitre plate shaker [Diagnostic Product Corporation, UK]
7. Dynatech MR5000 microtitre plate reader [Dynatech Laboratories, UK]

Method:

Preparations for PCR ELISA:

- On the day prior to each PCR ELISA experiment, the tubes for the experiment were labelled and the various buffers were aliquoted as set out in tables 2.5.1 to 2.5.4.
- At the start of the experiment, the working concentration of the anti-DIG-POD conjugate was prepared by diluting 1 volume of anti-DIG-POD conjugate in 99 volumes of its conjugate dilution buffer as set out in table 2.5.1. Care was taken to avoid foaming the mixture by gentle mixing and the solution was kept away from light by wrapping the universal container (or polypropylene tube) in aluminium foil. It was then left to stand at room temperature until required.

Table 2.5.1: Anti-DIG-POD working concentration

No. of samples	Vol. of conjugate buffer (ml)	Vol. of anti-DIG-POD (µl)
5	18.5	185
6	22.0	220
7	25.5	255
8	28.5	285

Table 2.5.2: Calculation of number of tubes and wells

No. of samples	No. of tubes	No. of wells	No. of strips (plates)
5	45	92	12 (1)
6	53	110	14 ($1^1/_6$)
7	61	126	16 ($1^1/_3$)
8	69	142	18 ($1^1/_2$)

No. of tubes = [No. of samples X 4 (*PCR*) x 2 (*dilutions*)] + 4 (*controls*) + 1 (*blank*)
No. of wells = [No. of tubes x 2] + 2 (*ABTS only*) [+ 2 (blanks on second plate)]
Each strip = 8 wells & each plate = 12 strips (96 wells)

Table 2.5.3 (a): IL-2, IL-10 & IFN-γ capture probe in hybridisation solution (100 ng/ml):

No. of samples (tubes)	Hybridisation solution (ml)	Capture probe (µl)
5 (11)	5.5	113
6 (13)	6.5	133
7 (15)	7.5	153
8 (17)	8.5	174

Table 2.5.3 (b): IL-4 capture probe in hybridisation solution (100 ng/ml):

No. of samples (tubes)	Hybridisation solution (ml)	Capture probe (μl)
5 (12)	6.5	133
6 (14)	7.5	153
7 (16)	8.5	173
8 (18)	9.5	194

Table 2.5.4: Substrate solution (1 ABTS tablet per 5 ml substrate buffer)

No. of samples	**Substrate buffer (ml)**	**ABTS tablets**
5	20	4
6	25	5
7	30	6
8	30	6

Dilution of PCR products:

- Next, 1 in 10 (1/10) and 1 in 40 (1/40) dilutions of the digoxigenin-labelled cytokine PCR products were prepared by diluting 5 μl of the digoxigenin-labelled cytokine PCR products in 45 μl of ddH_2O and 5 μl of 1/10 dilution in 15 μl of ddH_2O respectively. Each time the solutions were thoroughly mixed before use by vortexing. Preliminary PCR ELISA experiments using PCR products at various other dilutions (including neat concentration, 1 in 2, 1 in 4, 1 in 20 dilutions) have shown that the above dilutions were the appropriate dilution to ensure satisfactory readings by the MTP reader.

- However, with further experience of the PCR ELISA system used on the project samples and following the analysis of the data for the first 13 patients, we found that it was perfectly adequate to do only a single dilution for each digoxigenin-labelled cytokine PCR products and the 1 in 10 dilution was chosen for the remaining project samples since the spectrophotometric readings at this dilution were considerably higher than at the 1 in 40 dilution, thus aiding the comparison between the readings for each sample time point.

Denaturation of PCR products and capture probe hybridisation:

- 10 µl of the diluted digoxigenin-labelled cytokine PCR products and their negative controls were transferred to sterile 1.5 ml eppendorfs, serving as reaction tubes. 10 µl of sodium hydroxide (the denaturation solution provided in the kit) was used as the blanks for the MTP. 40 µl of sodium hydroxide was added to each reaction tube using the multipette with its combitips for rapid pipetting. The tubes' contents were mixed thoroughly by vortexing and then left to stand at room temperature for at least 10 minutes to denature the cytokine PCR products.

- During the denaturation step, the working solution of each cytokine biotinylated capture probe was prepared to a final concentration of 100 ng per ml of hybridisation buffer as set out in table 2.5.3. The MTP was prepared by mounting the correct number of streptavidin-coated MTP modules (8 wells per module) onto its frame.

- Following the period of denaturation, 450 µl of each cytokine biotinylated capture probe/hybridisation buffer was added to each corresponding cytokine PCR product reaction tube. Each reaction tube content was thoroughly mixed by vortexing and 200 µl of the reaction tube content per well was transferred to duplicate wells on the MTP. The position of the duplicate wells in the MTP corresponding to their reaction tubes on the plate was recorded. The MTP was then covered with self-adhesive cover foil (provided in the kit to prevent evaporation) and then incubated on a MTP shaker at 37 ^{0}C (the plate shaker was in the warm room) for 3 hours.

Detection of captured PCR products:

- At the end of the 3-hour incubation period, the solutions in the MTP were discarded by flicking the solutions in the wells off the plate and each well was washed 5 times with 250 µl of washing solution. The washing step was facilitated by using an 8-channel electronic pipette. At the last washing step, the washing solution was discarded and the MTP tapped dry on lint-free tissue paper. 200 µl of the working solution of the anti-DIG-POD conjugate was added to each well, the MTP again covered with the self-adhesive cover foil and then incubated on a microtitre plate shaker at 37 ^{0}C for 30 mins.

- During the incubation period for the anti-DIG-POD conjugate, the substrate was prepared by adding the ABTS tablets to the substrate buffer according to table 2.5.4. This was done 15 mins prior to use and the substrate was kept away from light by covering the universal container (or polypropylene tube) to minimise the natural extinction of the substrate with time and exposure to light. Following the incubation period, the solutions in the MTP was again discarded, the wells washed 5 times and tapped dry at the last washing step. 200 µl of the substrate was added to each well, the MTP was covered with the self-adhesive cover foil and aluminium foil to keep the MTP in the dark during the colour development phase. The MTP was again incubated on a MTP shaker at 37 ^{0}C.

- From 5 mins to 30 mins after the addition of the ABTS substrate, the MTP was removed at 5 mins intervals and read in a Dynatech MR5000 MTP reader set at dual wavelength mode, with the test filter set at 405 nm and reference filter set at 490 nm. The result was printed out for analysis.

2.6 Checking the integrity of the stored cDNA samples by PCR of the house-keeping β-globin gene

The integrity of the cDNA samples from each patient sample series in the project was verified by checking for the presence of the ubiquitous constitutionally expressed β-globin gene in each cDNA sample. This was done using a simple PCR for β-globin gene expression and resolving the PCR product band by agarose gel electrophoresis. As this check was performed after all the main experimental work was completed, both the integrity of the reverse transcription process as well as the intactness of the cDNAs following a variable period of storage at minus 80 ^{0}C were assessed.

The methodology for β-globin PCR was adapted from the standard operating procedure of the Molecular Laboratory, Wessex Immunology Service [Harris and Jones, 1997].

Consumables:

1. Ultra-violet light irradiated double-distilled water (ddH_2O)
2. Magnesium chloride ($MgCl_2$) at 25 mM concentration [Boehringer Mannheim, UK]
3. PCR reaction buffer **without** magnesium chloride, 10X concentration, containing 100 mM Tris-HCl, 500 mM KCl, pH 8.3 [Boehringer Mannheim, UK]
4. dNTP (deoxynucleotide triphosphate) mix, prepared by adding 12.5 µl of each stock Ultrapure dNTP [Pharmacia, UK] to 950 µl of ddH_2O, giving a concentration of 1.25 mM of each dNTP. This was used instead of the PCR DIG-labelling mix.
5. β-globin primers (300 ng) [R & D Systems, UK]:
   Sense sequence: 5'-CTG TGG GGC AAG GTG AAC G-3'
   Anti-sense sequence: 5'-CAA AGG ACT CAA AGA ACC TC-3'
6. *Thermus aquaticus* (*Taq*) polymerase at 5 U/µl [Boehringer Mannheim, UK]
7. autoclaved 1.5 ml eppendorfs [Greiner Labortechnik, UK]
8. autoclaved 0.75 ml PCR tubes [Greiner Labortechnik, UK]
9. Sterile pipette tips of various sizes [Greiner Labortechnik, UK]

10. Mineral oil [Sigma, UK]
11. HGT agarose [Sigma, UK] – 2% gel prepared by dissolving 4 g of agarose in 200 ml of 1X TAE (in a microwave), stained with 10 µl of ethidium bromide, and set in a gel tray with 22-well combs.
12. 50X TAE (Tris-Acetate-EDTA) - prepared with 121 g Tris [Sigma, UK], 28.55 ml glacial acetic acid [Merck, UK] and 50 ml 0.5 M EDTA pH 8.0, made up to 500 ml with ddH_2O and autoclaved. The 1X TAE was prepared by diluting the 50X TAE with ddH_2O.
13. 0.5 M EDTA pH 8.0 – 186 g/l EDTA [Merck, UK] with 20 g sodium hydroxide pellets [Merck, UK] to aid dissolution, the pH adjusted with 10 M sodium hydroxide [Merck, UK] and the solution autoclaved.
14. Ethidium bromide 10 mg/ml [Sigma, UK]
15. 6X stop mix - a mixture of 4 g sucrose [BRL, UK], 0.025 g bromophenol blue [Sigma, UK], 1.25 ml 50X TAE buffer diluted to 10 ml with ddH_2O. Working aliquots stored at 4 ^{0}C, stocks stored at minus 20 ^{0}C.
16. Molecular weight ladders VIII (19-1114 base pairs) and IX (72-1353 base pairs) [Boehringer Mannheim, UK]
17. Gel-loading pipette tips [Alpha laboratory, UK]

Equipment:

1. Elgastat Option 4 water purifier (for double-distilled water) [Elgaserve, UK]
2. Amplirad ultra-violet light cabinet [Genetic Research Instrumentation, UK]
3. Laminar airflow cabinet [MDH, UK]
4. Whirlimixer (vortex) [Jencons Scientific, UK]
5. Pipettes of various volume ranges (Gilson, Eppendorf, Biohit) [Anachem UK; Merck, UK; Alpha Laboratory, UK]
6. Perkin-Elmer thermal cycler [Perkin-Elmer, UK]
7. Gel tray, electrophoresis tank and power pack [Pharmacia, UK]
8. Ultra-violet light transilluminator [Genetic Research Instrumentation, UK]
9. DS 34 Polaroid direct screen instant camera and Polaroid ISO 3000 films [Genetic Research Instrumentation, UK]

Method:

Prior to the start of PCR experiments, sterile 1.5 ml eppendorfs, 0.75 ml PCR tubes and double distilled water were first irradiated with UV light for 10 to 15 mins to destroy any inadvertent contamination of the experiments with foreign DNA. The cDNA from each patient series and the reagents for PCR were thawed out in ice.

A master reagent mix was made for the β-globin PCR using the volumes of each reagent as set out in table 2.6.1. An extra aliquot for the negative control (using UV-irradiated double-distilled water) and an excess aliquot (to overcome the small but nevertheless unavoidable inherent inaccuracies arising from multi-aliquot pipetting), was included in the master reagent mix to ensure that the sufficient master reagent mix was available for each sample. Each reagent was vortexed immediately prior to aliquoting into the master reagent mix.

Each cDNA sample (thoroughly mixed first by pipetting in and out several times) was diluted down 1 in 10 (1 μl cDNA to 9 μl ddH_2O) first before aliquoting 10 μl of the diluted cDNA into a previously labelled 0.75 ml PCR tube. A negative control (10 μl ddH_2O instead of the diluted cDNA) was used for each run of the β-globin PCR. The *Taq* polymerase (kept at minus 20 ^{0}C) was added to the master reagent mix immediately before it was aliquoted into the PCR reaction tubes containing the diluted cDNA or ddH_2O. 90 μl of the PCR reagent mix (thoroughly mixed by vortexing prior to use) was added to each PCR tube containing ddH_2O or cDNA, making a total PCR reaction volume of 100 μl. Each PCR reaction was overlayed with 100 μl of mineral oil to prevent evaporation.

The PCRs were performed in the Perkin-Elmer thermal cycler using the program set out in table 2.6.2. A drop of mineral oil was also added to each slot (holding the PCR tube) in the metal block of the Perkin-Elmer thermal cycler before adding the PCR tubes to aid heat conduction. The PCR products were resolved in a 2% agarose gel. 10 μl of each PCR product was mixed with 2 μl of 6X stop mix and loaded into a well in the gel. The gel electrophoresis was run at 150 volts for about 1.5 hours with

a molecular weight ladder. A polaroid image of the UV-transilluminated agarose gel was performed to enable a visual assessment of the PCR product bands.

Table 2.6.1 - Master reagent mix for β-globin PCR

PCR reagents	**Volume per reaction (μl)**
UV-irradiated double distilled water	55.6
$MgCl_2$ (25 mM)	6.0
Boehringer 10X reaction buffer (without $MgCl_2$)	10.0
dNTP mix	16.0
Sense primer	1.0
Anti-sense primer	1.0
***Taq* polymerase (Boehringer)**	0.4

Table 2.6.2 - β-globin PCR program

• Denaturation: 94 ^{0}C for 5 minute
• 3 temperature PCR cycle with the following profile for 40 cycles: 94 ^{0}C for 2 minutes, 55 ^{0}C for 2 minutes, 72 ^{0}C for 3 minutes
• Final extension: 72 ^{0}C for 5 minutes
• Hold at 10 ^{0}C until the PCR products were stored at 4 ^{0}C

2.7 Statistical analyses of the data applied in this thesis

The statistical software "SPSS for Windows Release 7.5" [SPSS Inc, USA] was used to analyse the data from the preliminary cell separation experiments set out in chapter 3, all the clinical and laboratory data set out in chapter 5, and the RT-PCR ELISA data set out in chapter 6, generating the box plots and p values (when statistical tests were applied) in these chapters. The theoretical aspects of the statistical tests used in these analyses are briefly described in this section and a more comprehensive discourse on the subject can be obtained from "Practical Statistics For Medical Research" by Douglas G. Altman [Chapman & Hall, 1991]. The statistical tests used in the analyses of the data were chosen following consultation with a medical statistician of the University of Southampton.

When comparing groups of continuous data (numerical data which can take any value based on some form of measurement), both parametric tests (tests which make distributional assumptions on the population from which the sample is taken) and non-parametric tests (tests which do not make any distributional assumptions on the population from which a sample is taken) were used. The parametric tests used were the paired *t* test for comparison of means and the two sample *t* test for equality of means, and the non-parametric tests used were the Wilcoxon signed rank sum test for matched pairs and the Mann-Whitney U test. When comparing groups of categorical data (data based on the categories which an individual can be classified into), the Chi squared (χ^2) analysis of contingency tables or cross-tabulation was applied, with the Fisher's exact test being used as well when sample sizes were small (resulting in the expected frequencies within a contingency table being less than 5).

For each statistical test, the null hypothesis is that there is no difference between two sample means (or groups) and a p value of 0.05 or less is usually accepted as a reasonable probability for rejecting the null hypothesis, i.e. that the difference between the two sample means is statistically significant. The use of a statistical software program like "SPSS for Windows Release 7.5" gives much more precise p values than that obtainable from statistical tables described in the following sections.

In the results of every statistical tests used, a two-sided or two-tailed p value is always quoted.

Paired *t* test for comparison of means and two sample *t* test for equality of means

Both of these tests are based on the *t* distribution, described by W. S. Gossett, writing under the name of 'Student' in the early part of this century, hence it was sometimes also known as the Student's *t* distribution. He found that the mean of a sample from a Normal distribution with unknown variance has a distribution that is similar to, but not quite the same as, a Normal distribution. As the sample size increases the sampling distribution of the mean becomes closer to the Normal distribution. The *t* distribution has one parameter, a quantity called the degrees of freedom (df), which is the sample number minus one.

In both tests, the test statistic *t* is first calculated, which is the difference in the sample means divided by the standard error of the the sample means. For paired samples, the standard error of the sample means is calculated from the standard deviation of the differences between the paired observations divided by the square root of the sample number. For two independent samples, the standard error of the difference in sample means is the square root of the sum of the separate variances. The individual variance of each independent sample is calculated from the square of the standard deviation of the sample divided by its sample number. The p value is then read off from the t distribution table corresponding to the test statistic *t* and the degrees of freedom.

Wilcoxon signed rank sum test for matched pairs

This is a non-parametric equivalent of the paired *t* test. The Wilcoxon signed rank sum test, unlike the sign test, not only considers whether each observation in one sample is above or below the matched observation of the other sample, but also the magnitude of the differences. Ignoring the signs of the differences of the matched observations, each difference is first ranked in the order of its magnitude, and the

sums of the positive and negative ranks are then calculated. The sum of positive (or negative) ranks is then looked up against the corresponding sample size of matched observations in the Wilcoxon one sample (or matched pairs) test table and the corresponding p value read off.

Mann-Whitney *U* test

This is a non-parametric alternative to the *t* test for comparing data from two independent groups. This test requires all the observations to be ranked in the order of their magnitude as if they were from a single sample. Then the sum of the ranks in one group is calculated and the p value corresponding that sum and the respective sample sizes of the two groups are read off the Mann-Whitney test table. Note that the Mann-Whitney test is based on the assumption that there are no tied ranks. If tied ranks are present, statistical software like "SPSS for Windows Release 7.5" would automatically adjust for them.

The test statistic U is calculated from the equation: $U = n_1n_2 + \frac{1}{2}n_1(n_1+1) - T$, where n_1 and n_2 are the respective sample sizes and T is the sum of ranks in the smaller group. This test statistic U is the number of all possible pairs of observations comprising one from each sample for which one observation from one sample is less than the observation from the other sample. The estimated probability that a new observation from the first population will be less than a new observation sampled from the second population is U divided by the product of the sample sizes n_1 and n_2.

Chi squared (χ^2) test

This test is applied to the analysis of a contingency table or cross-tabulations of two categorical variables, so that possible associations between the variables can be evaluated. The test is based on the fact that when the null hypothesis is true (i.e., the two variables in the contingency table are unrelated), then the calculated test statistic χ^2 (described below) has a probability distribution called Chi squared distribution. The Chi squared distribution is the distribution of the square of a variable which has a standard Normal distribution on its own. The test involve the calculation of the

expected frequencies in each cell (the combination of each row and column categories) in the contingency table, which is the product of the relevant row and column totals divided by the sum of all the observed frequencies in the table (i.e. the sample size). The test statistic χ^2 is then calculated, which is the sum of the square of the difference between the observed and expected frequencies divided by the expected frequency in each cell in the contingency table. As the expected frequencies are calculated from the observed row and column totals, so the Chi squared test is 'conditional' on these totals. The test statistic χ^2 thus follows the Chi squared distribution with (r-1)(c-1) degrees of freedom under the null hypothesis, where r and c are the number of rows and columns respectively in the contingency table. By using this χ^2 test statistic and the appropriate degrees of freedom, the corresponding p value can be read off the χ^2 distribution table.

When sample sizes are small (i.e., when the expected frequencies in over 20% of the cells of the contingency tables are less than 5), the use of continuous Chi squared distribution to approximate frequencies introduces some bias into the calculation, so that the value of χ^2 tends to be a little too large. A continuity correction, consisting of moving the difference between observed and expected frequencies in each cell nearer to zero by half, is used to remove this bias, and in the context of 2 X 2 tables, the correction is known as Yates' correction.

For 2 X 2 tables with very small expected frequencies (less than 5), the Fisher's exact test was applied. This method is also based on the observed row and column totals and consists of evaluating the probability associated with all possible 2 X 2 tables which have the same row and column totals as the observed data, making the assumption that the null hyposthesis is true that the row and column variables are unrelated. The details of this test is given in chapter 10 of the book by Altman [1991].

Chapter 3: OPTIMISATION OF CELL SEPARATION METHODOLOGY

The cell separation method used for the project (as described in detail in section 2.1 of chapter 2) was chosen following preliminary experiments to evaluate the suitability of the various known methods described for separating lymphocytes from peripheral blood samples. The details of these preliminary experiments are described in this chapter.

3.1 Comparison of three combinations of cell separation techniques

This experiment was set up to compare the different cell separation techniques that could be used to separate lymphocytes from the buffy coat of mononuclear cells obtained following density gradient centrifugation of whole blood using Lymphoprep [Bøyum et al, 1991]. The three cell separation techniques of nylon wool column, monocyte adherence to plastics and panning were used in three different combinations to determine the best combination of cell separation techniques that would give the highest yield and purity of lymphocytes. A shortened version of the combined monocyte adherence/panning method was used [Stanciu et al, 1996].

Cell separation techniques

1. Nylon wool column: this is a non-specific physical method of cell separation based on the ability of phagocytic cells and cells with detectable surface membrane immunoglobulins to adhere onto a nylon wool column, while non-adherent cells would be eluted from the column [Eisen et al, 1972; Greaves and Brown, 1974]. This technique mainly removes the monocytes and B cells from the buffy coat of mononuclear cells.
2. Monocyte adherence to plastics: this is another non-specific physical method of cell separation based on the ability of monocytes to adhere onto the microscopic

irregularities in tissue culture grade petri dishes because of their phagocytic properties [Cline and Lehrer, 1968; Koller et al, 1973]. This method also removes platelets because of their inherent adherent properties upon activation.

3. Panning: this is a specific method of cell separation using mouse anti-human monoclonal antibodies to remove the monocytes, B lymphocytes and NK cells by negative selection onto plates coated with rabbit anti-mouse antibodies [Wysocki and Sato, 1978]. At the end of the panning step, the remaining mononuclear cells consist essentially of enriched T lymphocytes.

Materials and methods

The equipment and consumables used for this experiment were similar to those listed in section 2.1 (chapter 2) of the same subheading. Peripheral blood samples were taken from five healthy volunteers to test out the cell separation techniques. A total of 30 ml of blood was collected from each volunteer in three 10 ml vacutainer tubes containing lithium heparin as anticoagulants. An extra 2 to 3 ml of blood per volunteer was collected in a vacutainer tube (containing EDTA as anticoagulants) for cell counts and FACS. One 10 ml vacutainer tube of peripheral blood from each volunteer was used for each combination of cell separation experiment protocols (see below). All three combinations of cell separation protocols were performed in parallel on the same day. It was only possible to perform all three combinations of cell separation experiments on one volunteer at a time because of the length of time involved.

Cell counts of the whole blood and separated cells were performed using a Coulter Counter [Coulter, UK]. The purity of the separated cells was assessed in two ways. The first was using a flow cytometer to count the different mononuclear cell components in the separated cells. The second method was the visual assessment of the lymphocyte count in a cytospin preparation of the separated cells stained with haematoxylin and eosin dye. This visual assessment was performed by one of my supervisors (Dr J L Smith, Consultant Immunologist at Wessex Immunology Service, Southampton General Hospital).

The three combinations of cell separation techniques are detailed in the next three sections. For the description about Lymphoprep density gradient centrifugation, look under "principle" in section 2.1 of chapter 2.

Combination A – Lymphoprep density gradient centrifugation + nylon wool column

- Lymphoprep density gradient centrifugation: 10 ml of whole blood was carefully layered onto 10 ml of Lymphoprep in a 20 ml universal container. The tube was spun at 2500 rpm (1100g) for 20 mins in an MSE Mistral 3000i centrifuge at room temperature (set at 20 ^{0}C) and without any acceleration or brake settings.

- Nylon wool column: Following centrifugation, the buffy coat of mononuclear cells were carefully removed directly onto a nylon wool column (this was prepared by filling a sterile 20 ml syringe without its plunger loosely with nylon wool) using a Pasteur pipette. The column was then incubated at 37 ^{0}C for 30 mins.

  The non-adherent cells were then eluted from the nylon wool column with warm tissue culture medium (RPMI 1640 with HEPES, L-glutamine, sodium pyruvate and 10% FCS) and pelleted by centrifuging at 1500 rpm (400g) for 10 mins with maximum acceleration and brake applied. The supernatant was discarded and the cell pellet resuspended in 10 ml of warm tissue culture medium for cell count, FACS and cytospins.

Combination B – Lymphoprep density gradient centrifugation + nylon wool column + panning

- Lymphoprep density gradient centrifugation: 10 ml of whole blood was first diluted 1 in 1 with PBS /2% FCS in a sterile 50 ml polypropylene tube and then carefully layered into 3 sterile 15 ml conical-based polypropylene tubes containing 4 ml of Lymphoprep each. The tubes were spun at 2500 rpm (1100g) for 20 mins in an MSE Mistral 3000i centrifuge at room temperature (set at 20 ^{0}C) and without any acceleration or brake settings.

Following centrifugation, the buffy coat of mononuclear cells was carefully removed into a sterile 50 ml polypropylene tube using a Pasteur pipette. The mononuclear cells were then washed twice with PBS/2% FCS, the cells being pelleted by centrifuging at 1500 rpm (400g) for 10 mins (with maximum acceleration and brake applied), discarding the supernatant each time.

- Nylon wool column: The cell pellet was then resuspended in 5 ml of warm tissue culture medium and incubated in a nylon wool column at 37 ^{0}C for 30 mins. The non-adherent cells were then eluted from the nylon wool column with warm tissue culture medium and pelleted by centrifuging at 1500 rpm (400g) for 10 mins with maximum acceleration and brake applied. The supernatant was discarded and the cell pellet resuspended in 10 ml of warm tissue culture medium.

  2 ml of the cell suspension was removed at this stage for cell count, FACS and cytospins. The remaining cell suspension was by spun at 1500 rpm (400g) for 10 mins (with maximum acceleration and brake applied), discarding the supernatant.

- Panning: 500 μL of Dulbecco's PBS /5% FCS was first added to resuspend the cell pellet and 5 μL of each of the 4 mouse anti-human monoclonal antibodies (to CD 11b, 14, 16 & 19) [Becton Dickinson, UK; Serotec, UK] were added to the resuspended cells (table 2.1.1 in chapter 2 lists the different mononuclear cells which these monoclonal antibodies identify). The cell suspension was then incubated at 4 ^{0}C (by placing the 50 ml polypropylene tube containing the cell suspension in a bottle filled with ice) on a Denley Spiramix 5 rocker/roller [Denley Instruments, UK] for 30 mins to ensure thorough mixing.

  The excess antibodies were washed off twice with Dulbecco's PBS/5% FCS, centrifuging at 1500 rpm (400g) for 10 mins (with maximum acceleration and brake applied), discarding the supernatant each time. The pelleted cells were resuspended in 8 ml of Dulbecco's PBS/5% FCS and incubated on a Sterilin petri dish coated with rabbit anti-mouse antibodies for 1.5 hours at 4 ^{0}C (by placing

the plate in the fridge). The preparation of the rabbit anti-mouse antibodies coated Sterilin petri dish is described under "method" in section 2.1 of chapter 2.

At the end of the incubation period, the non-adherent cells were recovered onto a sterile 50 ml polypropylene tube by gentle washing of the Sterilin petri dish with Dulbecco's PBS/5% FCS using a Pasteur pipette and then pelleted by centrifuging at 1500 rpm (400g) for 10 mins (with maximum acceleration and brake applied), discarding the supernatant. The pelleted cells were finally resuspended in 5 ml of warm tissue culture medium for cell count, FACS and cytospins.

Combination C – Lymphoprep density gradient centrifugation + monocyte adherence + panning

- Lymphoprep density gradient centrifugation: This was performed as described in the density gradient centrifugation step of combination B.

- Monocyte adherence to plastics: Following the two washes of the buffy coat of mononuclear cells with PBS/2% FCS, the pelleted cells were resuspended in 8 ml of warm tissue culture medium. The cell suspension was then incubated on 2 tissue culture grade petri dishes in an incubator at 37 ^{0}C for 1 hour. The non-adherent cells were then removed onto a sterile 50 ml polypropylene tube by gently washing the petri dishes with warm tissue culture medium using the Pasteur pipette. The cells were again pelleted by centrifuging at 1500 rpm (400g) for 10 mins with maximum acceleration and brake applied. The supernatant was discarded and the cell pellet resuspended in 10 ml of warm tissue culture medium.

  2 ml of the cell suspension was removed at this stage for cell count, FACS and cytospins. The remaining cell suspension was by spun at 1500 rpm (400g) for 10 mins (with maximum acceleration and brake applied), discarding the supernatant.

- Panning: This was performed as described in the panning step of combination B.

Flow Cytometry

This section gives a brief and simplified description of flow cytometry. A thorough introduction to flow cytometry is described by Carter and Meyer [1994], from which most of the information in this section are based.

Flow cytometry is a technique for making rapid measurements on cells (or particles) as they flow in a fluid stream one by one through a sensing point. The measurements are made separately on each cell within the suspension in turn and not just as average values of the whole population. The addition of fluorescence analysis to the measurement of cellular parameters based on light scatter by the flow cytometer enables it to identify functional subpopulations of cells based on their cell surface and cytoplasmic determinants and receptors. These phenotypic markers are detected by monoclonal or polyclonal antibodies conjugated to a fluorochrome, a fluorescent dye which emits light of a certain wavelength when excited by light energy from a laser (acronym for '**l**ight **a**mplification by **s**timulated **e**misson of **r**adiation') beam in the flow cytometer. The two common fluorochromes in general use are phycoerythrin (PE) and fluorescein isothiocyanate (FITC).

Basically, flow cytometry involves the passage of a laser beam at right angle onto a moving stream of cells carried by a sheath of fluid in order to identify the different cell populations present within any given suspension of cells. The cells are specifically labelled by being incubated with fluorochrome conjugated antibodies. The effect of the laser beam on the individual cells passing through it is two fold. It is scattered in different directions by each cell in the stream and it causes the fluorochromes bound onto the cell surface via their conjugated antibodies to fluoresce. The scattered and fluorescent light generated by the cells passing through the laser beam are collected by photodetectors which convert the photon pulses into electronic signals which are analysed by a computer program to give the proportion of the different cell subpopulations in a given cell suspension.

The two main photodetectors in the flow cytometer are for the forward and side scatters. Through the use of a beam splitter, a dichroic mirror placed at 45^0 to the

incident beam, which reflects wavelengths shorter than 500 nm towards the side scatter detector while the longer wavelengths pass onto a second dichroic mirror. This separates the different wavelengths of the fluorescent light to pass through two separate sets of light filters onto two fluorescence detectors. A schematic diagram detailing the various components of a flow cytometer is shown in figure 3.1.1 .

The forward scatter detector "looks" at the scattered light gathered by the forward collection lens from approximately 1 to 20 degrees off the laser beam axis. It measures the angle of scatter of the laser beam when it strikes each cell. The size of the angle of scatter gives a relative measure of the size of the cells, i.e. the larger the angle, the larger the cell. The side scatter detector, on the other hand, "looks" at the part of the laser beam that had been reflected at right angle to the incident beam and it measures the degree of granularity of the cells. By combining the information about the size and granularity of the cells, the computer program is able to separate out the different cell types and give their proportion in a scatter diagram. Figure 3.1.2 gives an example of a computer-generated image of the distribution of the various cellular components of lysed whole blood based on light scatter only.

Figure 3.1.1 – Schematic diagram of flow cytometer

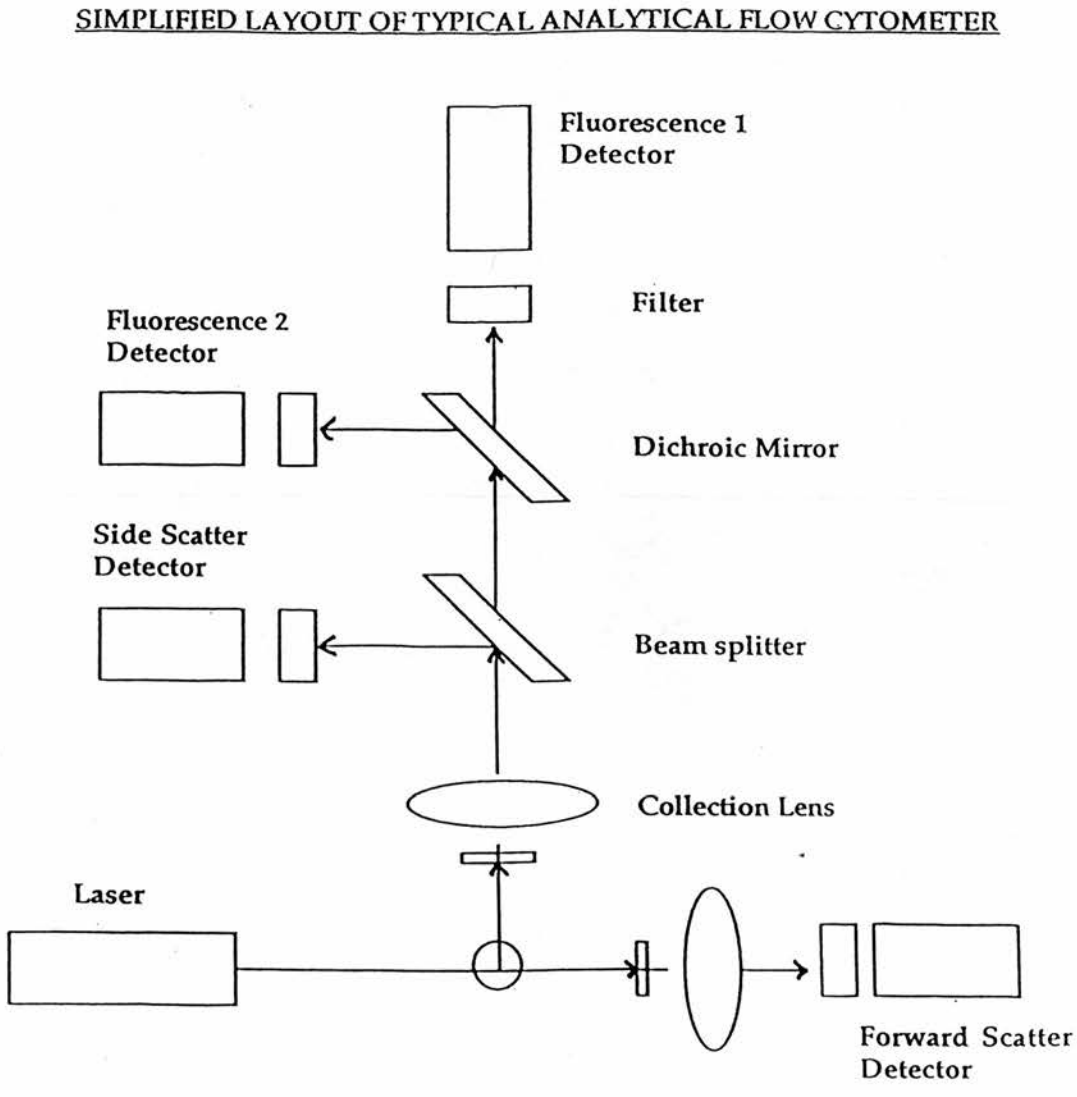
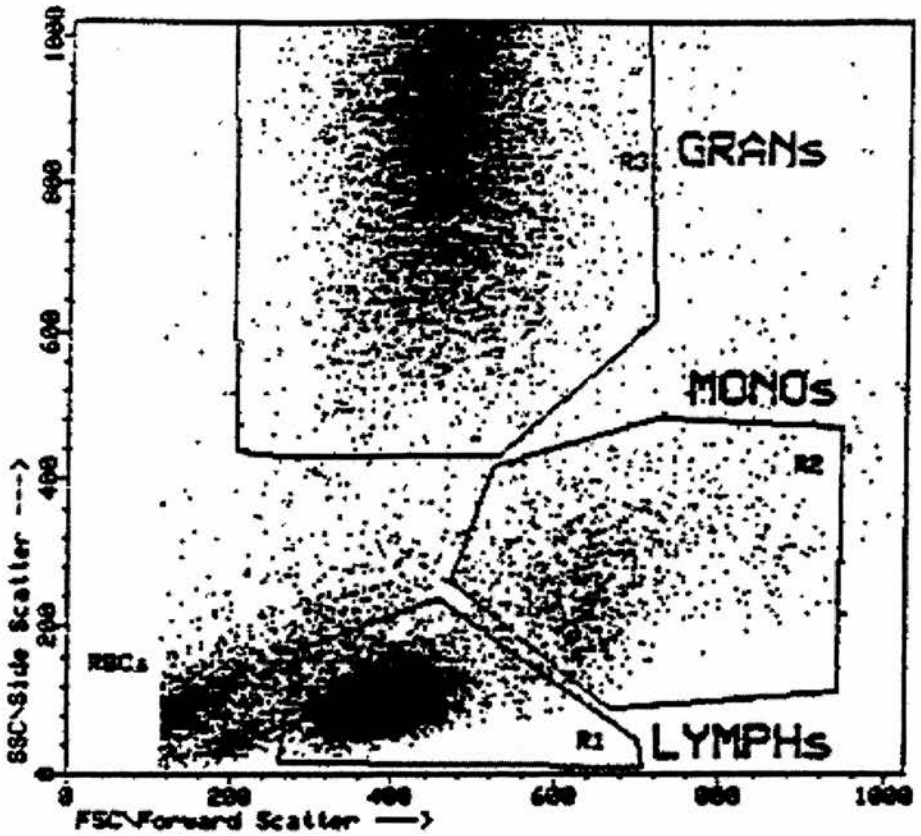


Figure 3.1.2 – Illustration of forward angle light scatter (X-axis) versus orthagonal (side) scatter (Y-axis) of lysed whole blood. The diagram shows the identification of 3 distinct cell populations based on light scatter only: R1 = Lymphocytes, R2 = Monocytes, R3 = Granulocytes. RBCs represent the area where unlysed red cells and debris will be found.



The resolution of the flow cytometer is related to the rate of aspiration of the sample by the machine. At low flow rate of about 12 µl/min, a very high resolution is possible and this is mainly used for cell sorting, while at high flow rate of about 60 µl/min, the resolution is lower and this is mainly used for phenotyping cells.

Method

Seven FACS tubes [Becton Dickinson, UK] were used for each sample of separated cell suspension. The control antibodies [Becton Dickinson, UK] used were unconjugated. The mouse anti-human monoclonal antibodies (mAb) to leucocyte cell markers [Becton Dickinson, UK; Serotec, UK] used for assessing the purity of the separated cells, and the combinations in which they were used and their respective conjugated fluorochromes, are shown in table 3.1.1. Table 3.1.2 sets out the principal cell type corresponding to its cell marker cluster of differentiation (CD) numbers.

Each mAb was diluted 1 in 40 from its stock solution prior to use. 20 μl of each diluted mAb was added to a 100 μl sample of separated cell suspension in each tube in accordance with the combinations set out in table 3.1.1. The antibodies were incubated with the separated cell suspensions for 30 mins at 4 ^{0}C before washing off the excess antibodies with 2 ml of PBS, centrifuging the tubes at 1500 rpm (400g) for 10 mins and discarding the supernatants. The cell pellets were then resuspended in the remaining volume in each tube by flicking the bottom of the tubes repeatedly.

Due to insufficient time to complete both the cell separation experiments and FACS data acquisition on the same day, the labelled cells in the FACS tubes were fixed in 1% paraformaldehyde in PBS to ensure that the labelled cells remained intact (though obviously no longer viable) for FACS data acquisition the following day. A Becton Dickinson FACSort [Becton Dickinson, UK] using the LYSIS II software was used. Whenever possible, 10000 cells were acquired from every sample for analysis. The data were stored in an optical disk for subsequent analysis.

Table 3.1.1 – Conjugated monoclonal antibody combinations used in the FACS tubes

Tube	**FITC**	**PE**
1	Control IgG	Control IgG
2	CD 16	-
3	CD 19	-
4	-	CD 14
5	-	CD 3
6	CD 4	CD 3
7	CD 8	CD 3

Table 3.1.2 – Cluster of differentiation (CD) and cell phenotype

CD	**Principal cell type**
3	All mature T lymphocytes
4	Helper T lymphocytes
8	Suppressor T lymphocytes
14	Monocytes
16	Natural killer cells
19	B lymphocytes

Cytospins

For each separated cell suspensions, cytospin slides were prepared by putting 2 to 3 drops of each separated cell suspension from a Pasteur pipette into the cytospin well with the Surgipath microscope slide and Shannon filter card [Life Sciences, UK] clamped to it. The loaded cytospin wells were then spun at 450 rpm for 10 mins in a Shannon Cytospin 2 [Life Sciences, UK]. The cytospin slides were air-dried before being stained with haematoxylin and eosin dye. A differential cell counts of each stained cytospin slides were made by one of my supervisors (Dr J L Smith, Consultant Immunologist, Southampton General Hospital).

Results

The cell counts and percentage mononuclear cell (MNC) loss following each cell separation technique are shown in Tables 3.1.3a and b. Note that the percentage cell loss in the tables takes into account the volume of separated cell suspension that were removed for cell counts, FACS and cytospins. A reading error in the cell count was obviously made by the Coulter counter for one of the samples from one volunteer (subject 2) because the total cell count at the end of panning was higher than before panning. Hence, this subject's cell counts was excluded from the analysis of cell loss. Table 3.1.4 shows the statistical analysis using the student's t-test for paired samples to compare the differences between the percentage of cell loss in the different combinations of cell separation techniques.

Tables 3.1.5a to f detail the percentages of the natural killer cells (CD 16), B lymphocytes (CD 19), monocytes (CD 14), T lymphocytes (CD 3), T-helper subset (CD 4) and T-suppressor subset (CD 8) in the separated cell suspensions. No FACS data were available for analysis for one volunteer (subject 1) because of technical errors made during acquisition of his FACS data.

Table 3.1.3a – Cell counts and % cell loss in combinations A and B

	Before separation	**After Nylon wool (A)**		**After Nylon wool (B)**		**After Panning (B)**	
Healthy subject	**MNC (10^6)**	**MNC (10^6)**	**% Cell Loss**	**MNC (10^6)**	**% Cell Loss**	**MNC (10^6)**	**% Cell Loss**
1	25.0	10.0	60.0	12.0	52.0	6.0	75.4
3	20.0	7.0	65.0	3.5	82.5	1.25	93.5
4	28.0	13.0	53.6	6.0	78.6	2.0	92.5
5	33.0	19.0	42.4	10.0	69.7	2.5	91.9
Average	**26.5**	**12.3**	**55.2**	**7.9**	**70.7**	**2.9**	**88.4**

Table 3.1.3b – Cell counts and % cell loss in combination C

	Before separation	**After Monocyte adherence (C)**		**After Panning (C)**	
Healthy subject	**MNC (10^6)**	**MNC (10^6)**	**% Cell Loss**	**MNC (10^6)**	**% Cell Loss**
1	25.0	12.5	50.0	9.2	62.4
3	20.0	12.5	37.5	7.5	57.1
4	28.0	15.0	46.4	7.5	70.0
5	33.0	17.0	48.5	10.5	64.5
Average	**26.5**	**14.3**	**45.6**	**8.7**	**63.5**

Table 3.1.4 – Differences in percentage cell loss (t-test for paired samples)

Paired differences of mean	**p-value**
Nylon wool (A) and Nylon wool (B)	NS
Nylon wool (A) and Monocyte adherence (C)	NS
Nylon wool (B) and Monocyte adherence (C)	NS
Nylon wool (A) and Panning (C)	NS
Nylon wool (B) and Panning (B)	0.01
Monocyte adherence (C) and Panning (C)	0.005
Panning (B) and Panning (C)	0.015

note: NS = not significant at $p<0.05$

Tables 3.1.5a – Percentage of monocytes (CD 14)

Healthy subject	**After Nylon wool (A)**	**After Nylon wool (B)**	**After Monocyte adherence (C)**	**After Panning (B)**	**After Panning (C)**
2	2.76	1.95	8.65	1.15	1.22
3	2.77	1.27	0.81	1.01	0.23
4	10.86	2.80	0.84	0.51	0.62
5	5.64	0.68	0.29	0.64	0.16
Average	**5.51**	**1.68**	**2.65**	**0.83**	**0.56**

Tables 3.1.5b – Percentage of natural killer cells (CD 16)

Healthy subject	After Nylon wool (A)	After Nylon wool (B)	After Monocyte adherence (C)	After Panning (B)	After Panning (C)
2	29.86	29.74	30.45	2.40	0.60
3	12.64	16.93	15.48	3.44	0.99
4	35.64	30.92	22.37	1.90	1.66
5	17.94	21.59	17.95	0.77	0.31
Average	**24.02**	**24.80**	**21.56**	**2.13**	**0.89**

Tables 3.1.5c – Percentage of B lymphocytes (CD 19)

Healthy subject	After Nylon wool (A)	After Nylon wool (B)	After Monocyte adherence (C)	After Panning (B)	After Panning (C)
2	7.39	1.84	6.29	0.21	0.73
3	5.98	2.89	7.03	1.99	1.98
4	11.54	2.01	9.20	0.41	1.11
5	6.45	2.74	3.42	0.65	0.44
Average	**7.84**	**2.37**	**6.49**	**0.82**	**1.07**

Tables 3.1.5d – Percentage of T lymphocytes (CD 3)

Healthy subject	After Nylon wool (A)	After Nylon wool (B)	After Monocyte adherence (C)	After Panning (B)	After Panning (C)
2	56.81	60.68	54.89	79.32	81.64
3	60.28	73.16	73.69	89.54	94.21
4	37.64	50.91	59.57	84.14	65.37
5	63.7	68.95	73.30	91.33	95.92
Average	**54.61**	**63.43**	**65.36**	**86.08**	**84.29**

Tables 3.1.5e – Percentage of T-helper subset (CD 4)

Healthy subject	After Nylon wool (A)	After Nylon wool (B)	After Monocyte adherence (C)	After Panning (B)	After Panning (C)
2	32.96	34.18	32.86	48.55	54.54
3	29.08	36.46	35.17	47.46	47.86
4	29.26	34.04	41.00	57.88	46.25
5	42.01	40.93	49.12	58.88	64.98
Average	**33.33**	**36.40**	**39.54**	**53.19**	**53.41**

Tables 3.1.5f – Percentage of T-suppressor subset (CD 8)

Healthy subject	After Nylon wool (A)	After Nylon wool (B)	After Monocyte adherence (C)	After Panning (B)	After Panning (C)
2	24.58	19.85	16.49	32.17	28.72
3	23.22	30.84	28.81	35.83	42.80
4	14.50	12.29	20.11	24.95	28.80
5	19.17	21.85	21.45	25.89	25.50
Average	**20.37**	**21.21**	**21.72**	**29.71**	**31.46**

The student's t-test for paired samples was also applied to compare the differences between the percentages of the different populations of mononuclear cells based on their phenotypic cell markers obtained following the three combinations of cell separation techniques (see Table 3.1.6).

Table 3.1.7 shows the percentage of lymphocytes as assessed by a visual differential count of the cytospin slides of the separated cell suspensions from the different

combinations of cell separation techniques. This serves as a comparison with the CD 3 count as obtained by FACS.

Using the student's t-test for paired samples, there were no significant differences between the mean percentages of lymphocytes in the cytospins except that between the cytospins for the separated cell suspensions at the end of the nylon wool and panning stages in combination B ($p=0.03$).

Table 3.1.6 – Paired differences of mean percentages of mononuclear cell components

Paired differences of mean	**p-value (t-test for paired samples)**					
	CD 16	**CD 19**	**CD 14**	**CD 3**	**CD 4**	**CD 8**
Nylon wool (A) and Nylon wool (B)	NS	0.033	NS	0.038	NS	NS
Nylon wool (A) and Monocyte adherence (C)	NS	NS	NS	NS	NS	NS
Nylon wool (A) and Panning (C)	0.021	0.015	NS	0.001	0.001	NS
Nylon wool (B) and Panning (B)	0.008	0.008	NS	0.009	0.009	0.035
Monocyte adherence (C) and Panning (C)	0.008	0.014	NS	0.026	0.027	0.021

note: NS = not significant at $p<0.05$

Table 3.1.7 – Percentage of lymphocytes in cytospin differential counts

Healthy subject	After Nylon wool (A)	After Nylon wool (B)	After Monocyte adherence (C)	After Panning (B)	After Panning (C)
1	94.3	92.9	98.6	97.4	99.5
2	96.8	89.4	88.8	95.6	94.7
3	82.8	95.4	95.6	98.8	96.8
4	89.9	88.3	92.5	90.9	93.0
5	98.8	98.9	99.3	99.0	97.1
Average	**92.5**	**93.0**	**94.9**	**96.3**	**96.2**

Discussion

In terms of percentage cell loss at the end of each cell separation technique, there were no significant differences between the two non-specific physical techniques which removes mainly monocytes. However, the addition of a second specific technique to the cell separation combinations B and C resulted as expected in a further significant cell loss (an extra 18%) by the negative selection of the panning technique. The mean total percentage cell loss at the end of combination B was significantly higher than for combination C. Interestingly, despite the significant further cell loss by adding the panning step to monocyte adherence in combination C, the mean total percentage cell loss for combination C was not significantly different from that for combination A, which only uses a single physical step of cell separation to improve the lymphocyte yield (tables 3.1.3a and b, 3.1.4).

There were no significant differences in the efficiency of removing monocytes between the nylon wool column and monocyte adherence to plastics methods. Although the addition of panning to the other two techniques further reduced the percentage of CD 14 cells further, this was not significant statistically (table 3.1.5a). However, panning significantly reduced the percentages of NK cells (CD 16) and B

cells (CD 19) in the separated cell suspensions (tables 3.1.5b and c), thus improving the overall yield of T lymphocytes and its subsets (tables 3.1.5d to f, 3.1.6).

Overall, the visual assessment of the cytospins of the separated cell suspensions suggested that a high lymphocyte yield (in excess of 90%) was obtainable from all the cell separation techniques used (table 3.1.7), although it is obviously impossible to separate out the NK cells and B cells visually in a haematoxylin and eosin slide without resorting to immunohistochemical techniques.

In conclusion, combination C of cell separation techniques (combining monocyte adherence to plastics with panning) gives the best yield of T lymphocytes as well as the best purity in terms of low percentages of the other "contaminating" mononuclear cell (CD 14, 16 and 19). This was therefore the cell separation method chosen for the project.

3.2 Comparing full-time with half-time cell separation

This set of experiments was set up to see if the total time taken to perform the combined monocyte adherence/panning cell separation method could be reduced further without affecting the percentage of cell loss or the yield and purity of the T lymphocytes obtained. The principal reason for trying to shorten the total time taken to perform the combined monocyte adherence/panning cell separation method is to decrease the time of in vitro manipulations of the T lymphocytes and thus minimise the risk of activating these cells.

Method

The experiments were conducted in two separate batches. In the first batch, 14 stable renal transplant patients visiting the outpatient department for routine follow-up assessment were recruited. They were randomly assigned to two equal groups (full-time and half-time cell separation). 15 to 20 ml of peripheral blood in lithium

heparin vacutainer tubes and 2 to 3 ml of blood in an EDTA vacutainer tube were collected from each patient.

In the second batch, parallel experiments to compare full-time and half-time cell separations were performed on 2 healthy volunteers. Two 10 ml lithium heparin vacutainer tubes of peripheral blood (one each for the full-time and half-time experiments) and 2 to 3 ml of blood in an EDTA vacutainer tube were collected from each volunteer. In all subjects, the EDTA blood was used for cell count only.

The cell separation methodology was as described for combination C in section 3.1 with modifications made in the duration of the incubation period for monocyte adherence and panning steps. The incubation period for monocyte adherence was 1 hour for the full-time experiment and 30 mins for the half-time experiment, while the incubation period for panning was 1.5 hours for the full-time experiment and 45 mins for the half-time experiment. Both batches of experiments were performed in the transplant laboratory in Portsmouth.

From each separated cell suspension, cell counts by Coulter counter in the Haematology department of St Mary's Hospital, Portsmouth, were performed. The samples for FACS data acquisition were sent to Wessex Immunology Service, Southampton General Hospital but because of logistical reasons, the FACS data acquisition were usually performed (by Wessex Immunology Service staff) the following day.

Results

The cell counts and percentage mononuclear cell (MNC) loss for the first batch of experiments are shown in table 3.2.1 and the flow cytometry data giving the yield and purity of T lymphocytes are detailed in table 3.2.2.

No data are available for the percentage of B cells (CD 19) in the separated cell suspensions as there were problems with the quality of the conjugated monoclonal antibodies for phenotyping CD 19 making it impossible to gate the B cell population

for analysis. For the full-time separation group, no FACS data were available for one of the patients (no. 4).

The results for the second batch of experiments are shown in table 3.2.3 (cell counts and percentage mononuclear cell loss) and table 3.2.4 (FACS data on T lymphocytes yield and purity).

No statistical test was applied on any of these results.

Table 3.2.1 – Cell counts and % cell loss in full-time and half-time cell separation

Patient	**Whole blood**	**After 1 hr monocyte adherence & 1.5 hr panning**		**Patient**	**Whole blood**	**After 30 mins monocyte adherence & 45 mins panning**	
	MNC (10^6)	**MNC (10^6)**	**% Cell Loss**		**MNC (10^6)**	**MNC (10^6)**	**% Cell Loss**
1	13.3	4.0	69.9	8	47.5	22.0	53.7
2	45.6	12.0	73.7	9	28.5	4.0	86.0
3	45.6	14.0	69.3	10	43.5	30.0	31.0
4	28.5	12.0	57.9	11	66.5	36.0	45.9
5	41.8	14.0	66.5	12	26.6	10.0	62.4
6	23.8	8.0	66.4	13	30.4	18.0	40.8
7	26.0	16.0	38.5	14	34.2	18.0	47.4
Av	**32.1**	**11.4**	**63.2**	**Av**	**39.6**	**19.7**	**52.4**

Table 3.2.2 – FACS data in full-time and half-time cell separation

Patients	After 1 hr monocyte adherence & 1.5 hr panning			Patients	After 30 mins monocyte adherence & 45 mins panning		
	CD 14 (%)	CD16 (%)	CD 3 (%)		CD 14 (%)	CD 16 (%)	CD 3 (%)
1	13.01	0.82	74.68	8	17.40	0.23	79.12
2	4.66	1.56	94.20	9	1.35	1.42	97.68
3	3.06	0.62	89.55	10	1.66	0.17	89.03
4	-	-	-	11	2.79	0.71	95.75
5	9.33	1.72	87.42	12	4.23	0.95	78.60
6	5.41	0.41	51.02	13	15.98	0.72	72.89
7	6.39	0.41	95.11	14	2.66	0.27	79.23
Av	**6.98**	**0.92**	**82.00**	**Av**	**6.58**	**0.64**	**84.61**

Table 3.2.3 – Cell counts and % cell loss in full-time and half-time cell separation

Healthy subjects	Whole blood	After 1 hr monocyte adherence & 1.5 hr panning		Healthy subjects	Whole blood	After 30 mins monocyte adherence & 45 mins panning	
	MNC (10^6)	MNC (10^6)	% Cell Loss		MNC (10^6)	MNC (10^6)	% Cell Loss
1	22.0	10.0	54.5	1	22	10.0	54.5
2	18.0	10.0	44.4	2	18	10.0	44.4
Av	**20.0**	**10.0**	**49.5**	**Av**	**20.0**	**10.0**	**49.5**

Table 3.2.4 – FACS data in full-time and half-time cell separation (healthy subjects)

Subjects	After 1 hr monocyte adherence & 1.5 hr panning				After 30 mins monocyte adherence & 45 mins panning			
	CD 14 (%)	CD 16 (%)	CD 19 (%)	CD 3 (%)	CD 14 (%)	CD 16 (%)	CD 19(%)	CD 3 (%)
1	2.02	0.14	2.64	84.57	1.93	0.28	2.27	87.05
2	3.62	0.16	2.98	86.01	2.69	0.14	1.37	81.86
Av	**2.82**	**0.15**	**2.81**	**85.29**	**2.31**	**0.21**	**1.82**	**84.46**

Discussion

The average percentage mononuclear cell loss in the first batch of cell separation experiments on peripheral blood from stable renal transplant patients was less for the half-time cell separation than for the full-time cell separation, but the T lymphocyte yield and purity in the two groups were almost the same. While the reduced incubation time for the two cell separation techniques may result in a decreased cell loss, this was not at the cost of a poorer T lymphocyte yield or purity.

Similarly, results from the second batch of cell separation experiments almost mirror the results of the first batch, with the average percentage of mononuclear cell loss being the same for the full-time and half-time cell separation groups, and the T lymphocyte yield and purity in the two groups being very close.

Based on these results, the half-time incubation period for monocyte adherence and panning was adopted as the project protocol. This much shortened cell separation method reduced the time of in vitro manipulations of the T lymphocytes and consequently the risk of activating the cells.

Chapter 4: OPTIMISATION AND VALIDATION OF RT-PCR ELISA PROTOCOLS

This chapter reports on a series of experiments which were conducted to test and validate the molecular protocols used in the project. The first section describes an investigation into the optimum magnesium chloride concentration for use in the polymerase chain reaction protocols. The next two sections look at the use of the project's RT-PCR ELISA protocol to assay the gene expression levels of cDNA samples from patients recruited for the project, firstly to check for reproducibility of the results, and secondly to assess the relationship between the level of gene expression of the cDNA samples and the resultant quantity of PCR products. The final section details a comparison of the results of the RT-PCR ELISA protocol when applied to neat and diluted cDNA samples in order to determine whether similar patterns of sequential cytokine gene expression are demonstrable independent of the absolute amount of starting cDNA.

4.1 Optimising the magnesium chloride concentration for polymerase chain reaction (PCR)

The PCR protocols used in the project were modified from the original protocols developed by the Molecular Immunology Laboratory of the Department of Medicine, University of Southampton. These original PCR protocols had been extensively tested and used by the research staff of the Department of Medicine.

The first modification in the PCR protocols is the use of a different *Taq* DNA polymerase. The Department of Medicine used *Taq* DNA polymerase manufactured by Promega in their protocols while the *Taq* DNA polymerase used for this project was purchased from Boehringer Mannheim (chosen because this *Taq* DNA polymerase is routinely used in the Molecular Biology Laboratory of Wessex Immunology Service, where this project was undertaken). The second modification

is in the amount of magnesium chloride ($MgCl_2$) used in the PCR protocols. The amount of $MgCl_2$ in a PCR is important since the activity of the *Taq* DNA polymerase requires free magnesium on top of that bound by template DNA, primers, and dNTPs [Innis and Gelfand, 1990]. Moreover, it may also affect any of the following: primer annealing, strand dissociation temperatures of both template and PCR product, product specificity, formation of primer-dimer artifacts, and enzyme activity and fidelity. It was therefore prudent to check that the same $MgCl_2$ concentration used in the original PCR protocols with Promega *Taq* DNA polymerase would remain optimal when used with Boehringer *Taq* DNA polymerase instead, before the PCR protocols were applied to the project samples.

For granzyme B (GrB) and fas ligand (FasL) PCR amplification, it was necessary to determine the optimal $MgCl_2$ concentrations since these were not given in the paper by Strehlau et al [1997] and PCR for these gene products had not been validated in Southampton prior to this project.

Consumables and equipments:

Most of the consumables and equipments listed in section 2.6 (chapter 2) were used for the optimisation experiment (and the other experiments described in this chapter), with some changes listed below:

1. IL-2, IFN-γ, IL-4 and IL-10 cytokine primers (see section 2.4, chapter 2)
2. 0.2 ml thin-walled PCR tubes/caps (strip of 8 tubes/caps each) [Advanced Biotechnologies, UK]
3. Perkin-Elmer GeneAmp System 9600 thermal cycler [Perkin-Elmer, UK]

Method:

The PCR methodology used in this experiment is set out fully in section 2.4 (chapter 2). The cDNA samples used for this experiment were obtained by reverse transcription of total RNA extracted from the purified peripheral T lymphocytes of stable renal transplant patients (from the experiments described in section 3.2 of the previous chapter) or renal failure patients. Three different cDNA samples were used for each set of magnesium optimisation experiments. The magnesium chloride

optimisation experiments were carried out for all the cytokines and cytotoxic T-cell markers studied in the project.

The master reagent mix used for the magnesium chloride ($MgCl_2$) optimisation PCR experiment is summarised in table 4.1.1. Five aliquots of master reagent mix were prepared for each magnesium chloride concentration, this includes an extra aliquot for the negative control (using UV-irradiated double-distilled water) and another aliquot as the excess, to take into account the inherent inaccuracies (small but nevertheless unavoidable) resulting from multi-aliquot pipetting, so that sufficient volume of master reagent mix was available for each sample. The magnesium chloride concentrations were varied between 1.0 mM and 2.5 mM for IL-2, IFN-γ, IL-4 and IL-10, and between 1.0 mM and 3.0 mM for IL-13, GrB and FasL, both sets at 0.5 mM increments. It was only possible to do a limited magnesium chloride optimisation experiment for IL-5 because preliminary experiments showed that it was completely undetectable in any of the patient's or healthy volunteer's cDNA, but was only detectable in one cDNA sample from stimulated peripheral lymphocytes kindly supplied by the Department of Medicine. Due to this lack of cDNA, only 1.0 mM and 2.0 mM concentrations were used to assess the preferable magnesium chloride concentration for IL-5 PCR.

The PCRs were performed in a Perkin-Elmer 9600 thermal cycler using the program and corresponding optimal annealing temperatures set out in tables 2.4.4 and 2.4.5 (section 2.4, chapter 2). The PCR products were resolved in a 2% agarose gel. 10 μl of each PCR product was mixed with 2 μl of 6X stop mix and loaded into a well in the gel. The gel electrophoresis was run at 150 volts for about 1.5 hours with a molecular weight ladder. A polaroid image of the UV-transilluminated agarose gel was performed to enable a visual assessment of the PCR product bands.

Table 4.1.1 - Master reagent mix for magnesium chloride optimisation RT-PCR

$MgCl_2$ Concentration (mM):	**1.0**	**1.5**	**2.0**	**2.5**	**3.0**
ddH_2O (μl)	60.0	57.5	55.0	52.5	50.0
25 mM $MgCl_2$ (μl)	5.0	7.5	10.0	12.5	15.0
Boehringer 10X reaction buffer (without $MgCl_2$) - 2.5 μl*	12.5	12.5	12.5	12.5	12.5
dNTP mix (1.25mM) – 4.0 μl*	20.0	20.0	20.0	20.0	20.0
Sense primer (10 μM) - 1.5 μl*	7.5	7.5	7.5	7.5	7.5
Anti-sense primer (10 μM) - 1.5 μl*	7.5	7.5	7.5	7.5	7.5
Taq* polymerase (Boehringer) - 0.2 μl	1.0	1.0	1.0	1.0	1.0

Notes: * this is the volume per aliquot; all volumes are in μl (a total of 5 aliquots of master reagent mix were prepared)

Results

The result from the magnesium chloride optimisation experiment is summarised in table 4.1.2, which compares the magnesium chloride concentrations from the original RT-PCR protocols and what was considered optimum for the Boehringer *Taq* DNA polymerase from the assessment of the gel image.

The criteria for the optimum magnesium chloride concentration is that concentration which gives the brightest specific PCR product band on the agarose gel image and/or having the least amount of non-specific bandings. All the agarose gel images from the optimisation experiment are shown in figures 4.1.1 to 4.1.8. As a comparison, the agarose gel image of β-globin RT-PCR for a selection of patients is shown in figure 4.1.9 (the details of the RT-PCR protocol is described in section 2.6).

Table 4.1.2 – Optimum $MgCl_2$ concentrations – comparison of original protocol concentrations with experimental findings

	Original protocol (mM)	**Experimental findings (mM)**
IL-2	1.0	2.0
IFN-γ	1.0	2.0
IL-4	1.0	2.5
IL-10	2.0	2.5
IL-5	1.0	1.0
IL-13	2.0	2.0
GrB	not available	1.0
FasL	not available	1.0

Discussion

The results of these experiments have demonstrated similar optimum $MgCl_2$ concentrations to the original PCR protocols for some cytokines (IL-5 and IL-13) but different optimum $MgCl_2$ concentrations in others (IL-2, IFN-γ, IL-4 and IL-10). As explained previously, no comparison was possible for GrB and FasL. These experimentally derived optimum $MgCl_2$ concentrations were used for all experimental PCR protocols in this project.

Figure 4.1.1 – IL-2 (PCR product size 255 base pairs)

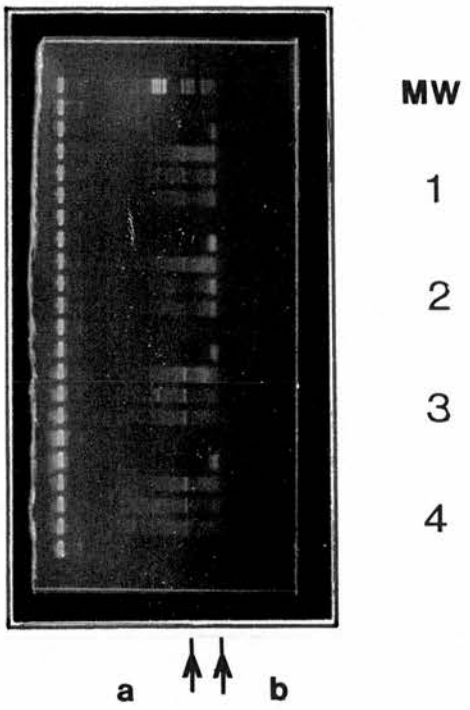
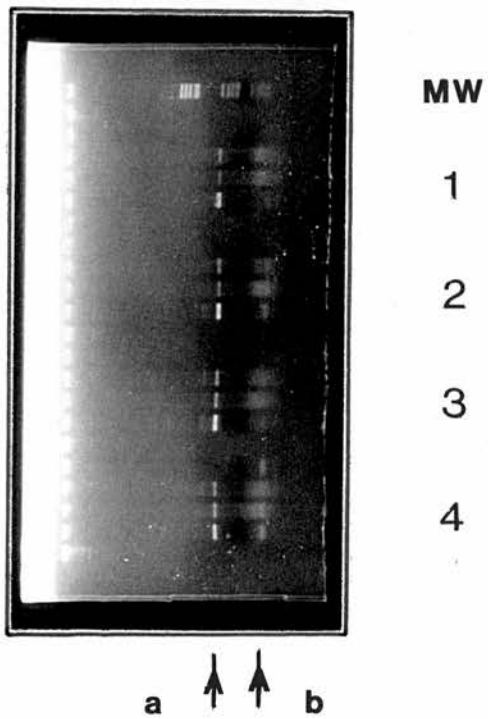


Figure 4.1.2 – IFN-γ (PCR product size 270 base pairs)



In both gel images: MW = molecular weight marker V (22 fragments, pBR322 DNA cleaved with *Hae* III); each group consist of a negative control and 3 different cDNA samples; 1 = 1.0 mM, 2 = 1.5 mM, 3 = 2.0 mM, 4 = 2.5 mM.
Arrow "a" marks the PCR product bands and arrow "b" marks the primer bands

Figure 4.1.3 – IL-4 (PCR product size 449 base pairs)

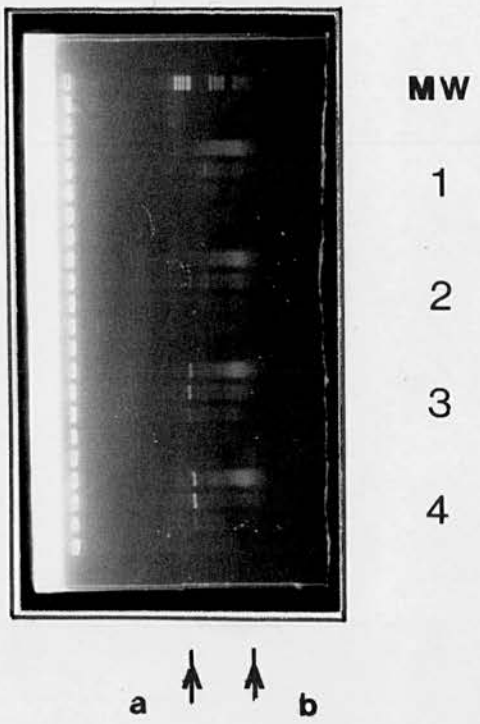
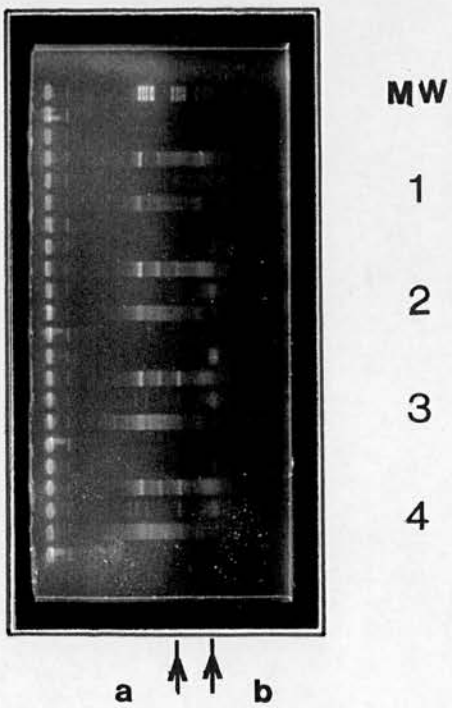


Figure 4.1.4 – IL-10 (PCR product size 231 base pairs)



In both gel images: MW = molecular weight marker V (22 fragments, pBR322 DNA cleaved with *Hae* III); each group consist of a negative control and 3 different cDNA samples; 1 = 1.0 mM, 2 = 1.5 mM, 3 = 2.0 mM, 4 = 2.5 mM.
Arrow "a" marks the PCR product bands and arrow "b" marks the primer bands

Figure 4.1.5 – IL-5 (PCR product size 257 base pairs)

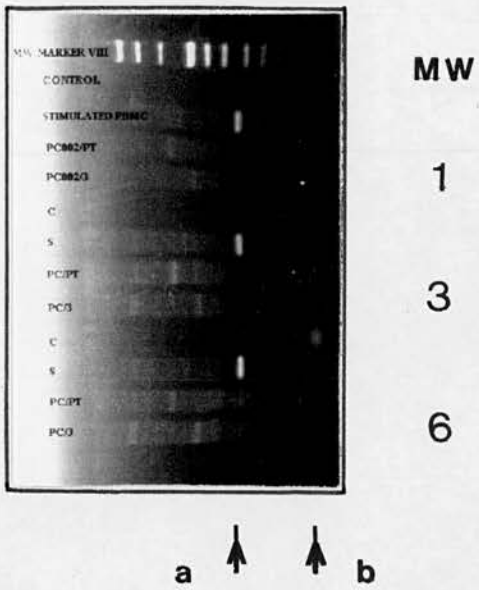
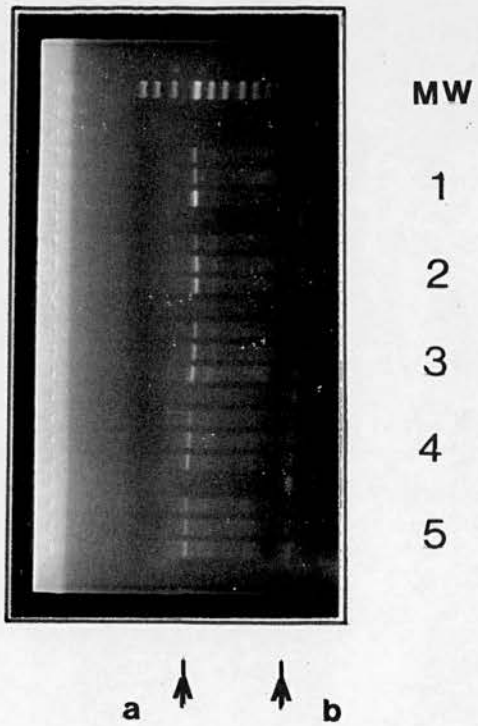


Figure 4.1.6 – IL-13 (PCR product size 500 base pairs)



In both gel images: MW = molecular weight marker VIII (17 fragments, mixture of pUCBM21 DNA, cleaved with *Hpa* II and pUCBM21 DNA, cleaved with *Dra* I and *Hind* III); each group consist of a negative control and 3 different cDNA samples; 1 = 1.0 mM, 2 = 1.5 mM, 3 = 2.0 mM, 4 = 2.5 mM, 5 = 3.0 mM, 6 = 2.0mM and double amount of primers.
Arrow "a" marks the PCR product bands and arrow "b" marks the primer bands

Figure 4.1.7 – GrB (PCR product size 431 base pairs)

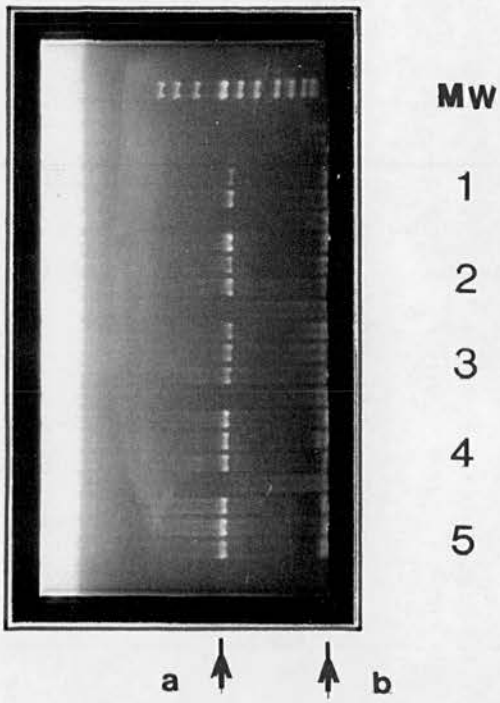
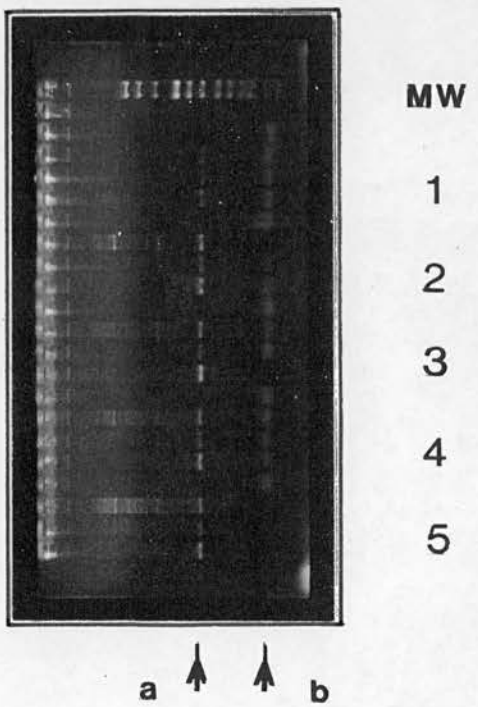
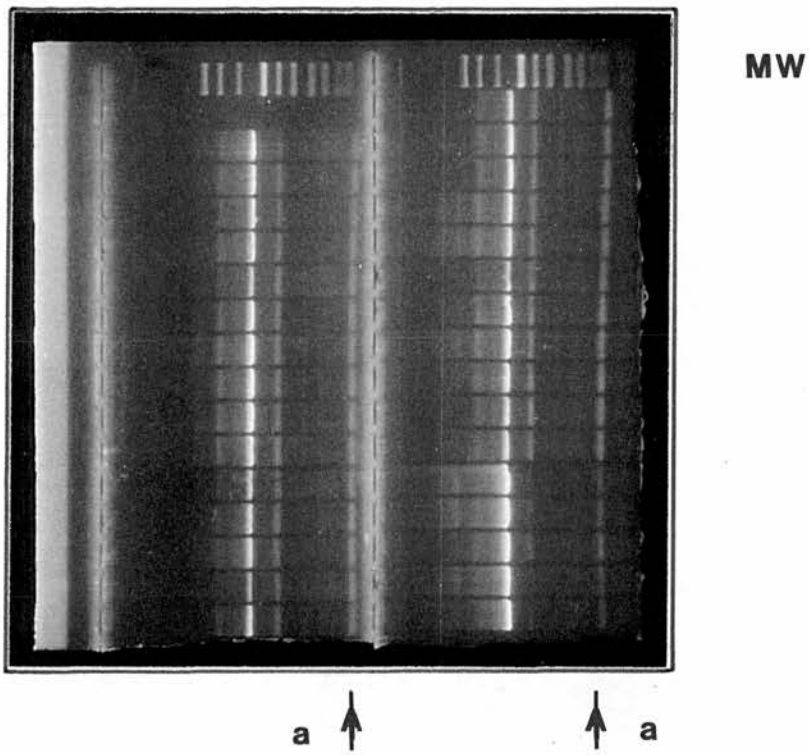


Figure 4.1.8 – FasL (PCR product size 301 base pairs)



In both gel images: MW = molecular weight marker VIII (17 fragments, mixture of pUCBM21 DNA, cleaved with *Hpa* II and pUCBM21 DNA, cleaved with *Dr*a I and *Hind* III); each group consist of a negative control and 3 different cDNA samples; 1 = 1.0 mM, 2 = 1.5 mM, 3 = 2.0 mM, 4 = 2.5 mM, 5 = 3.0 mM.
Arrow "a" marks the PCR product bands and arrow "b" marks the primer bands

Figure 4.1.9 – β-globin (PCR product size 97 base pairs)



In this gel image: MW = molecular weight marker VIII (17 fragments, mixture of pUCBM21 DNA, cleaved with *Hpa* II and pUCBM21 DNA, cleaved with *Dr*a I and *Hind* III); a single negative control and β-globin bands for 31 samples from 7 different patients are shown here.
Arrow "a" marks the PCR product bands. The primer bands lies outside the gel image.

4.2 Testing the reproducibility of the RT-PCR ELISA protocol

This experiment was conducted to investigate the reproducibility of the project's entire molecular protocol (combining RT-PCR with PCR ELISA detection of the PCR products) for detecting IL-2, IFN-γ, IL-4 and IL-10 cytokine gene expression (4 out of the 8 gene products investigated in the project).

Method

The cDNA samples of all the sampling time points from one of the patients (a non-rejector) recruited for the project were used in this experiment. The combined RT-PCR ELISA protocol (sections 2.4 and 2.5) was repeated on the same time series of cDNA samples from the same patient on 3 separate occasions. There was a 2 weeks' gap between the first and second runs of the RT-PCR ELISA protocol, and a 3 weeks' gap between the second and third runs.

Results

Figures 4.2.1 to 4.2.4 expressed the three runs of the reproducibility experiments graphically for IL-2, IFN-γ, IL-4 and IL-10 series respectively. In each graph, the amount of PCR products (reflected by the level of absorbance units of the products detected spectrophotometrically by the PCR ELISA technique) is set out along the y-axis, while the different sampling time points (/PT = pre-transplant, /2 = early post-transplant, /3 = 1 week post-transplant, /4 = 2 weeks post-transplant, /5 = 4 weeks post-transplant) are shown along the x-axis. R1, R2 and R3 denotes the results from first, second and third runs respectively obtained from applying the project's RT-PCR ELISA protocol on the same patient's cDNA samples on 3 separate occasions.

Figure 4.2.1

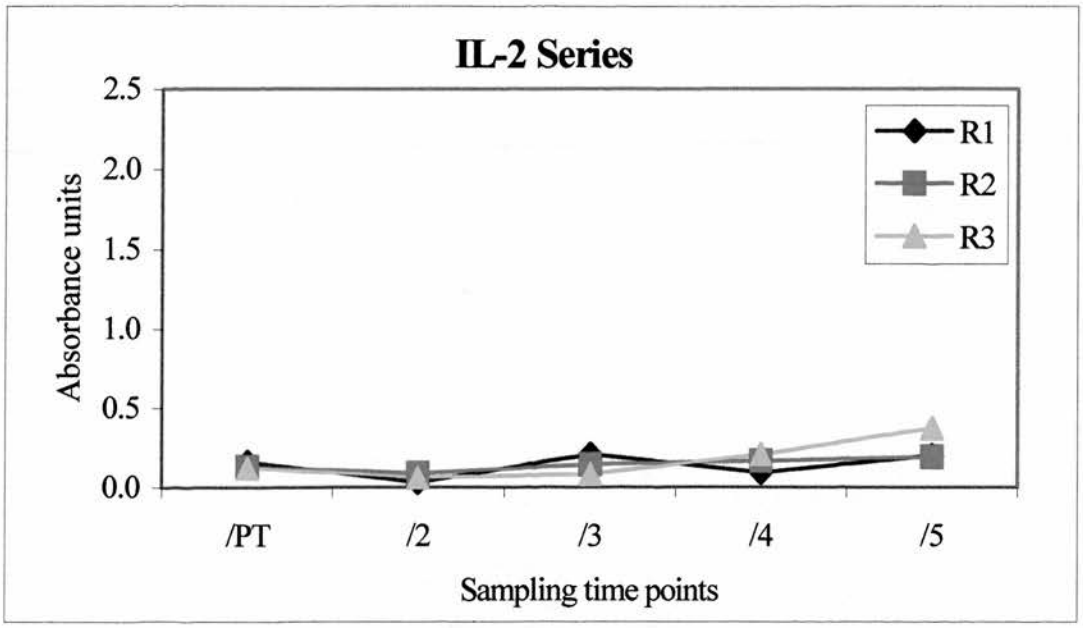
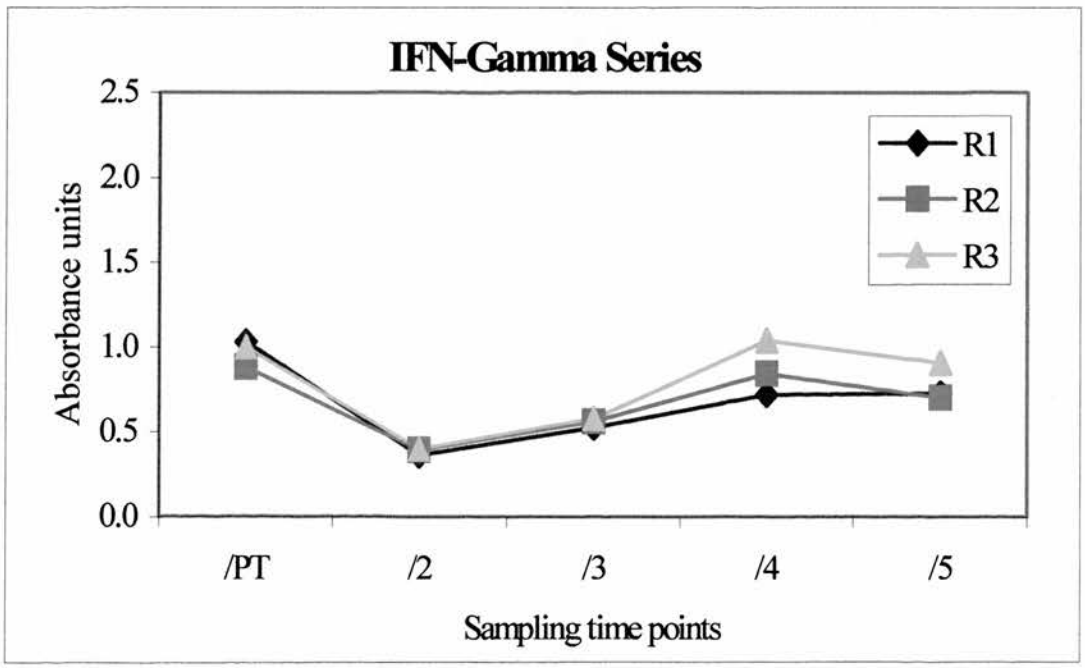


Figure 4.2.2



Figures 4.2.1 and 4.2.2 show the RT-PCR ELISA profiles for IL-2 and IFN-γ respectively on 3 separate runs (R1, R2 and R3) for the same patient.

Figure 4.2.3

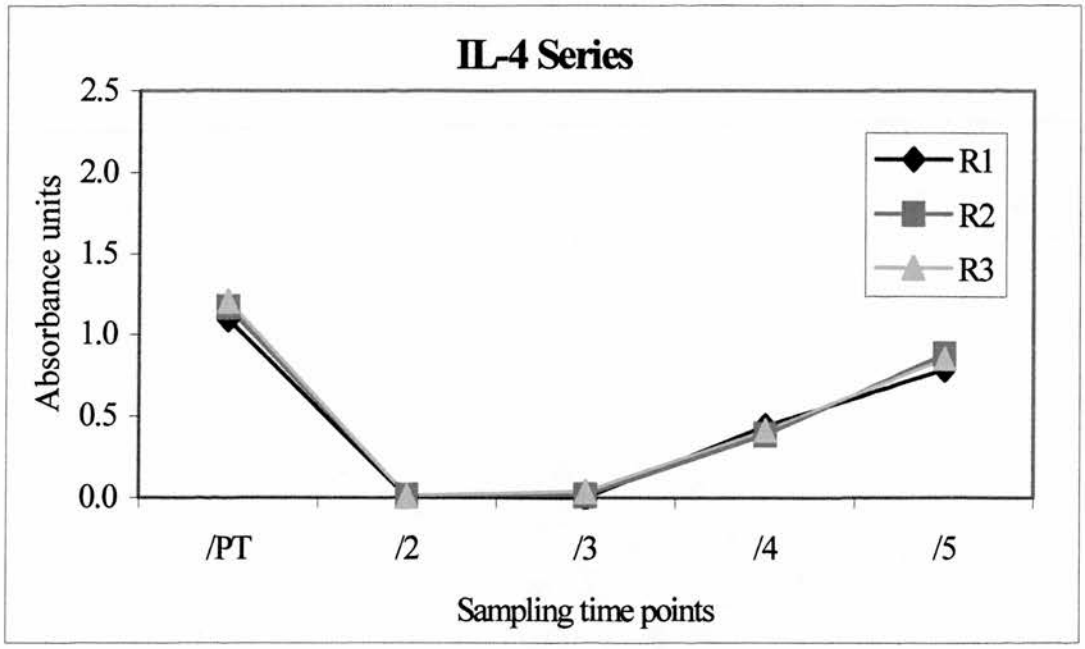
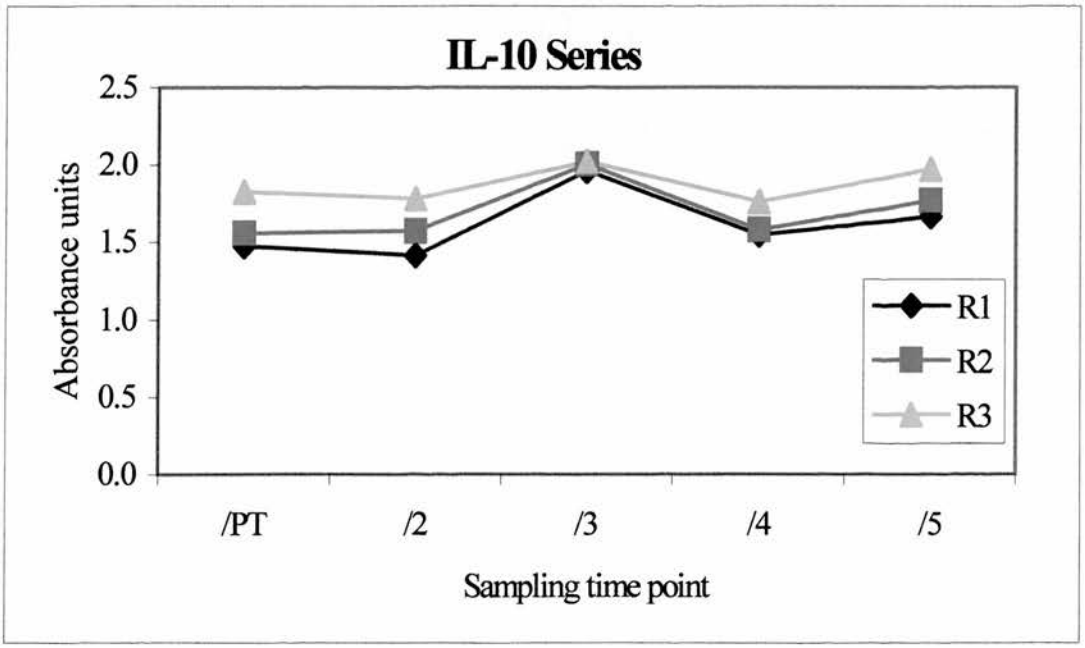


Figure 4.2.4



Figures 4.2.3 and 4.2.4 show the RT-PCR ELISA profiles for IL-4 and IL-10 respectively on 3 separate runs (R1, R2 and R3) for the same patient.

Discussion

The results of these experiments indicate that within the limits of biological variability, the project's RT-PCR ELISA protocol is able to produce highly reproducible results when applied repeatedly to a patient series of samples. This is true both for high level of cytokine gene expression (IFN-γ, IL-10 and most of IL-4 series) as well as low level of cytokine gene expression (IL-2 series and time points 2 and 3 of IL-4 series).

4.3 Dilution experiment to assess the relationship between the level of gene expression of the cDNA samples and the resultant quantity of PCR products

Dilution experiments were set up to investigate the relationship between the starting amounts of cytokine cDNA and the resultant quantity of IL-2, IFN-γ, IL-4 and IL-10 PCR products, as measured in absorbance units spectrophotometrically by PCR ELISA, reflecting the levels of cytokine gene expression in the peripheral T lymphocyte samples of the patients recruited in the project. It is important for the quantity of PCR products to be directly related to the level of cytokine gene expression (i.e. an increase in PCR products implies an increase in cytokine gene expression, and vice versa) so that the PCR ELISA data obtained in the project patients can be interpreted in a semi-quantitative manner.

Method

For these experiments, 15 cDNA samples taken from 9 patients in the project (some patients contributing samples from different sampling time points) were selected on the single criterion of high levels of PCR products from using neat concentrations (i.e. undiluted) of cDNA for the PCR. This was to ensure that a reasonable level of PCR products would be obtained when the neat concentrations of cDNA were diluted down for the experiments. For each cytokine studied, 6 cDNA samples were used for the dilution experiments.

The RT-PCR ELISA protocol (sections 2.4 and 2.5) was applied to the cDNA samples chosen for the experiments. The cDNA samples were used neat, and at two other dilutions (diluting the cDNA with UV-irradiated double-distilled water). For IFN-γ, IL-4 and IL-10 PCRs, the two dilutions of cDNA prepared were 1 in 5 and 1 in 20, but for IL-2 PCR, 1 in 2 and 1 in 8 dilutions were made instead. These lower dilutions were used for IL-2 because of the generally low levels of IL-2 PCR products generated from RT-PCR of neat cDNA samples (see appendices 8 and 9).

Results

The results of the dilution experiments for IL-2, IFN-γ, IL-4 and IL-10 are expressed graphically in figures 4.3.1 to 4.3.4 respectively. The amount of PCR products is set out along the y-axis in absorbance units while the three different dilutions of cDNA are shown along the x-axis. The 15 cDNA samples were labelled A to O. It can be seen that some cDNA samples were used for more than one dilution experiment.

For IL-2, all diluted cDNA samples (both 1 in 2 and 1 in 8 dilutions) have lower amount of PCR products compared with that from their corresponding neat cDNA, except for one sample where the 1 in 2 dilution have a slightly higher amount of PCR products than its corresponding neat cDNA sample (sample L, figure 4.3.1). For the other 3 cytokines with known high levels of PCR products from neat cDNA, in 14 (77.8%) out of 18 samples (5 for IFN-γ, 6 for IL-4 and 3 for IL-10), the amount of PCR products were higher from 1 in 5 dilution of cDNA than from neat cDNA. With further dilution of the cDNA down to 1 in 20, the resultant amount of PCR products in 15 (83.3%) of 18 samples (5 for IFN-γ, 4 for IL-4 and 6 for IL-10) were lower than that from neat cDNA.

The unusually low amount of IL-4 PCR products from neat cDNA seen in sample F (figure 4.3.3) clearly suggests an inefficient PCR (for whatever reasons), since the same sample did produce much higher amount of IL-4 PCR products in previous experiments.

Discussion

The generally low level of IL-2 expression was seen in the rapid fall in the amount of PCR products with a small dilution of the starting cDNA down to 1 in 8 compared with the other 3 cytokines which were a lot more highly expressed than IL-2.

For the highly expressed cytokines (IFN-γ, IL-4 and IL-10), it is interesting to note that with the initial 1 in 5 dilution of cDNA, the resultant amount of PCR products actually rose above that from neat cDNA in 77.8% of samples, and only fell to below the PCR product levels from neat cDNA at the greater dilution of 1 in 20 (in 83.3% of samples). As the cDNA samples chosen for the dilution experiments were all known to result in a large amount of PCR products for these cytokines, reflecting a lot of gene transcripts present in the starting cDNA samples, this interesting anomaly may be explained by an inhibition of the PCR caused by a reduced efficiency of cDNA template annealing with its primers when excessively large amounts of gene transcripts were present in the starting cDNA samples [Wang et al, 1989].

The reason for this is that for an exponential increase in PCR products to be possible, the amount of primers for each cytokine must be in a vast excess over that for its template, so that primer-template annealing is greatly in excess of template-template annealing. At high concentrations of cDNAs, this will become progressively less true. With a little dilution of these highly concentrated cDNA samples, the efficiency of cDNA template binding by its primers improved, resulting in a larger amount of PCR products. With a higher dilution of the starting cDNA, the confounding factor of template-template annealing is no longer a problem, so the amount of PCR products then start to fall as one would expect.

These results also demonstrate the highly sensitive nature of PCR as a technique in detecting the presence of cytokine cDNA gene transcripts. The disproportionately much greater change in cDNA concentration compared to its corresponding PCR product levels suggests that the relationship between the amount of PCR products is not always related to the amount of cDNA gene transcripts in a linear fashion. It is

Figure 4.3.1

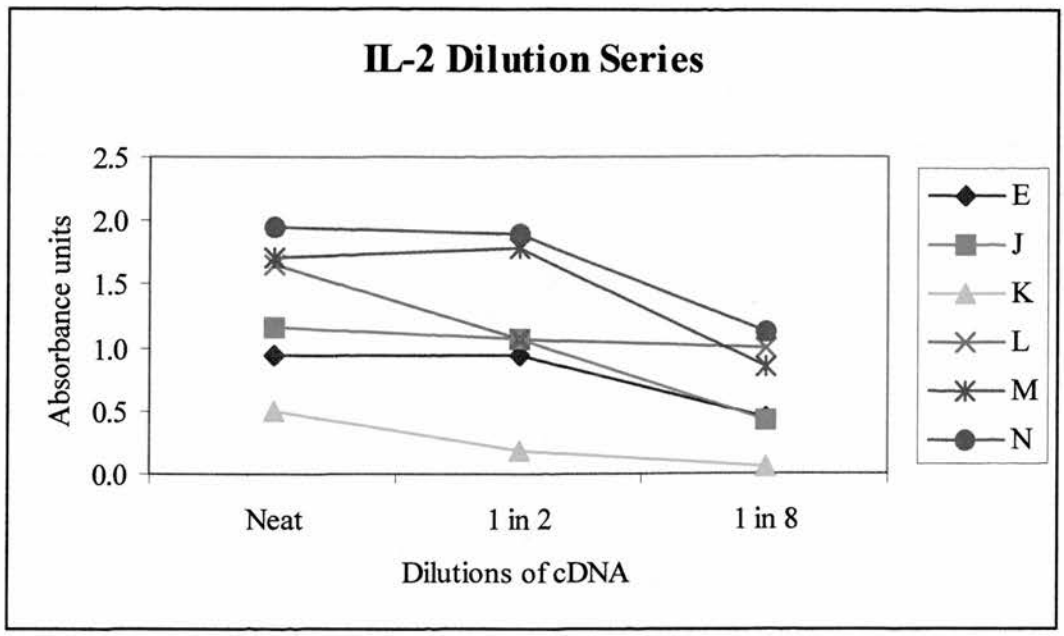
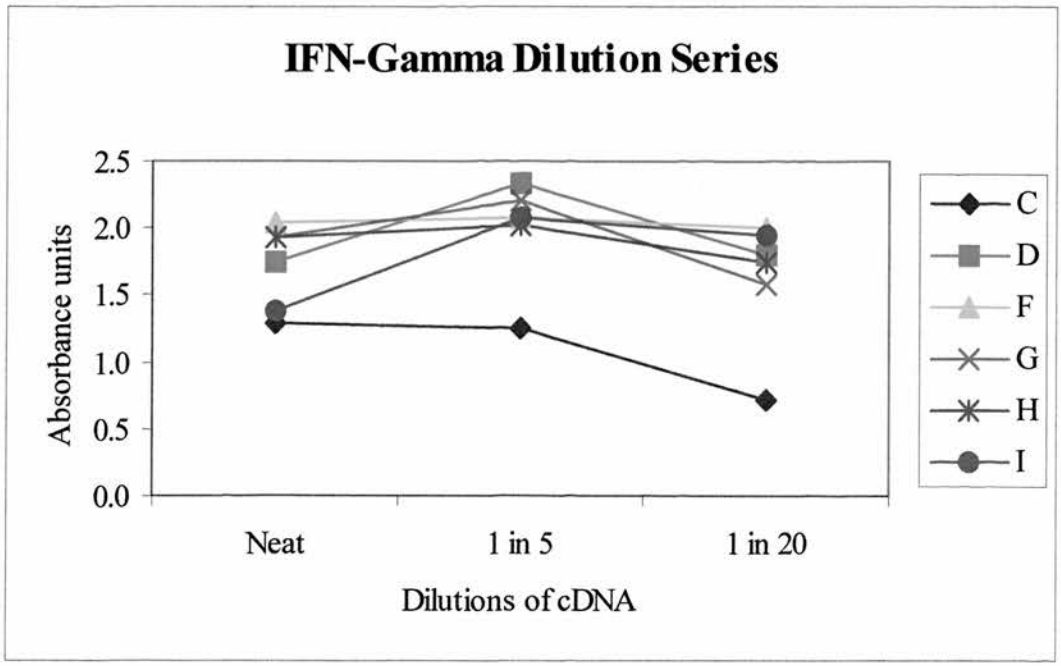


Figure 4.3.2



Figures 4.3.1 and 4.3.2 show the dilution series for IL-2 and IFN-γ respectively. Each graph (labelled by a capital letter) represents the dilution series for one sample of cDNA.

Figure 4.3.3

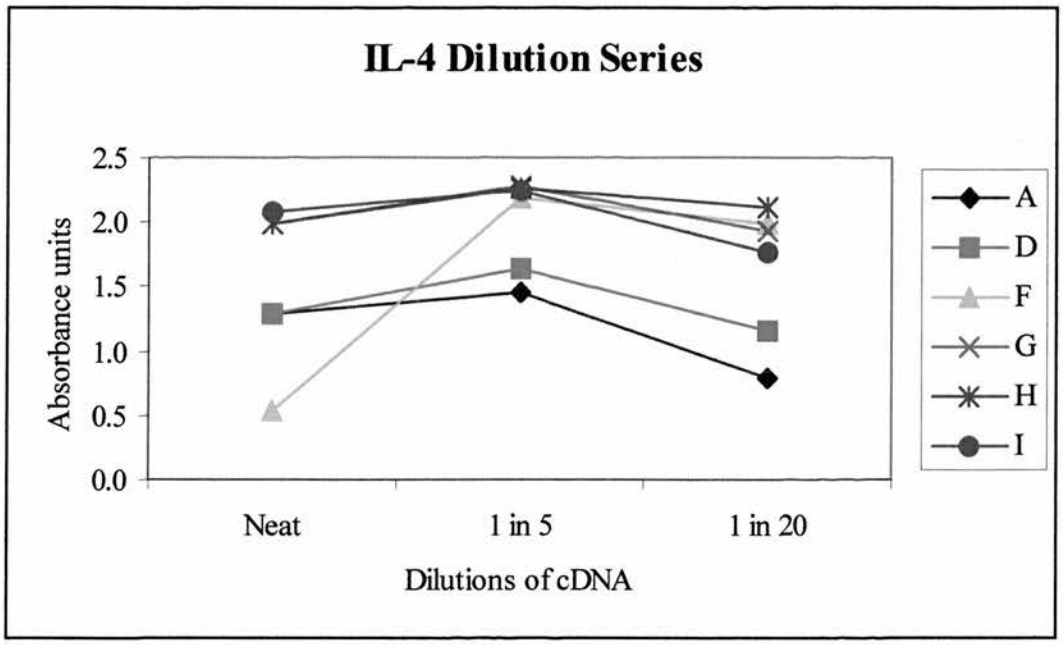
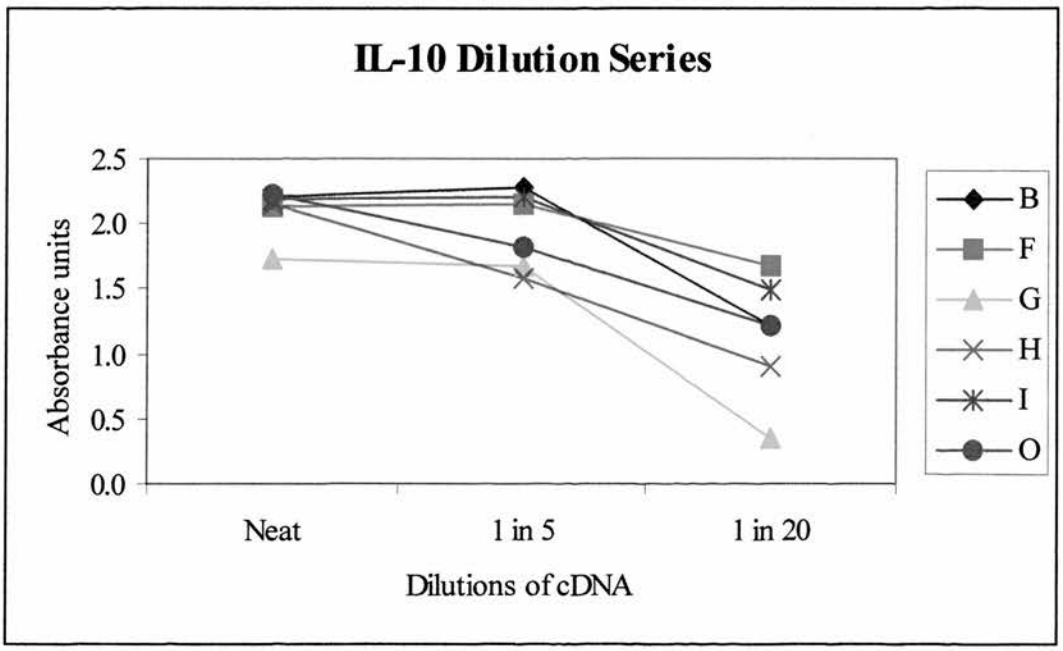


Figure 4.3.4



Figures 4.3.3 and 4.3.4 show the dilution series for IL-4 and IL-10 respectively. Each graph (labelled by a capital letter) represents the dilution series for one sample of cDNA.

therefore only reasonable to compare the results of the PCR ELISA data at the different time points for each patient in a semi-quantitative manner.

4.4 Would a diluted cDNA series of samples gives the same trend of changes as the same but undiluted cDNA series of samples?

In view of the results of the previous dilution experiments showing a slight increase in the amount of PCR products with the initial dilution of cDNA (those with high levels of cytokine gene transcripts) before the expected fall in amounts of PCR products with further dilution of the starting cDNA, the obvious question is whether this finding would pose any problems in term of the trend of changes in a series of samples from the same patient if neat cDNAs were used for the PCR instead of diluted ones (i.e. is the trend independent of the absolute amount of starting cDNA for any given time point)? This experiment was therefore conducted to answer this very question.

Method

The complete series of cDNA samples from two patients (both non-rejectors) were used in this experiment. The project's RT-PCR ELISA protocols for IL-2, IFN-γ, IL-4 and IL-10 were applied to each patient's cDNA samples used neat and at 1 in 10 dilution. Each experiment (either with neat or 1 in 10 dilution of cDNA) were performed at different times.

Results

The results of the experiments are expressed graphically in figures 4.4.1 to 4.4.4 for IL-2, IFN-γ, IL-4 and IL-10 series respectively.

Figure 4.4.1a – IL-2 series (patient 1)

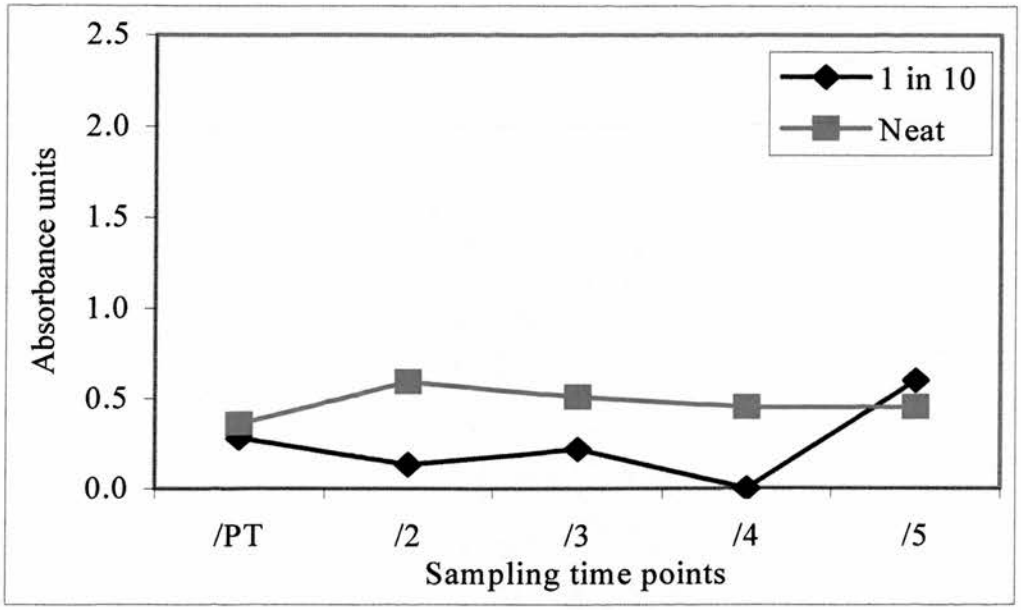
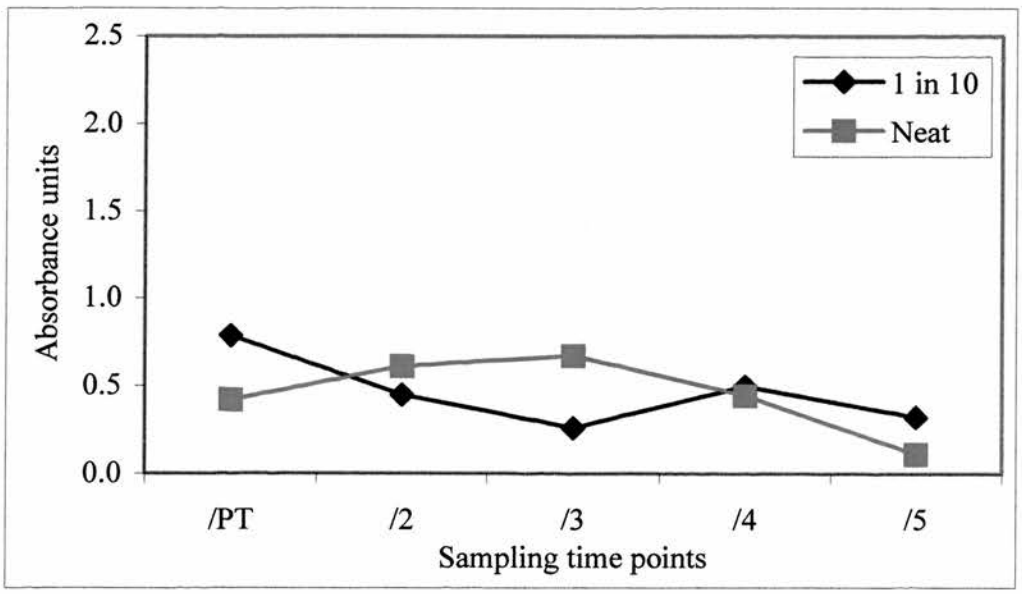


Figure 4.4.1b - IL-2 series (patient 2)



Figures 4.4.1a and 4.4.1b show the RT-PCR ELISA profile for IL-2 in two patients using neat and 1 in 10 dilution of cDNA (as labelled within each figure).

Figure 4.4.2a – IL-4 series (patient 1)

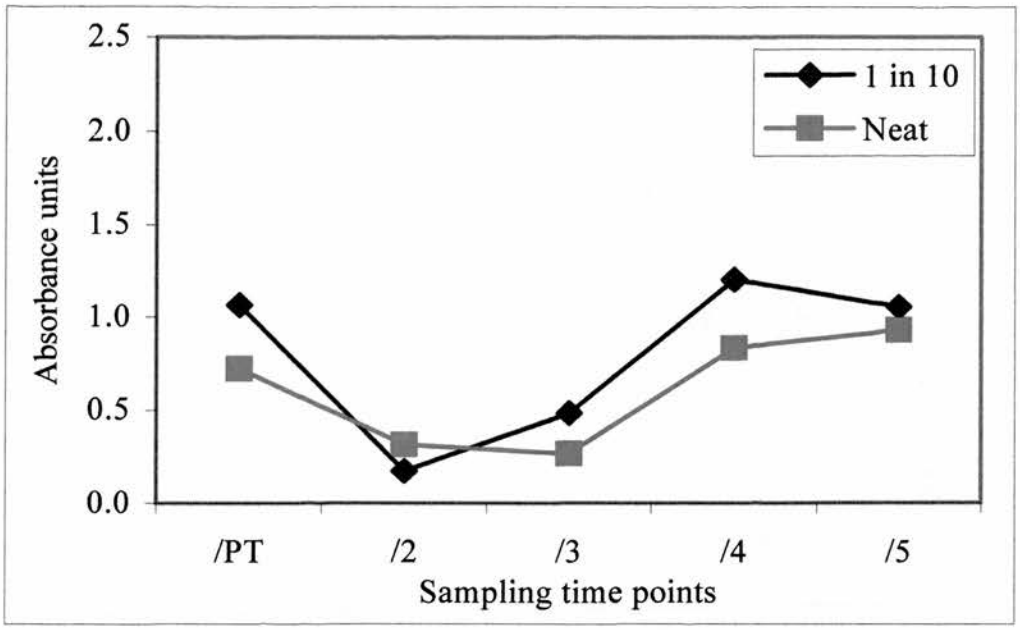
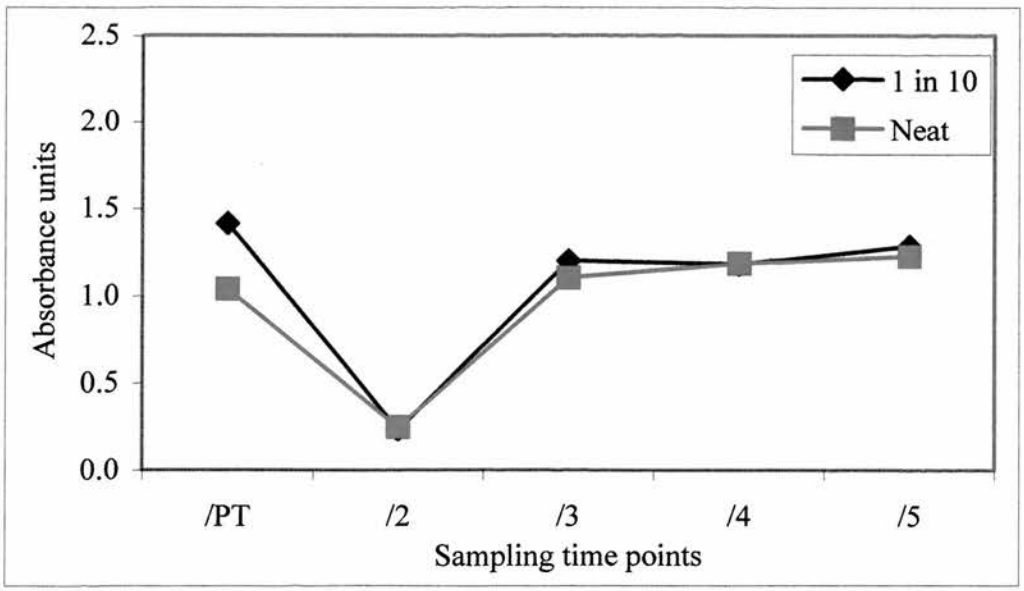


Figure 4.4.2b – IL-4 series (patient 2)



Figures 4.4.2a and 4.4.2b show the RT-PCR ELISA profile for IL-4 in two patients using neat and 1 in 10 dilution of cDNA (as labelled within each figure).

Figure 4.4.3a – IFN-γ series (patient 1)

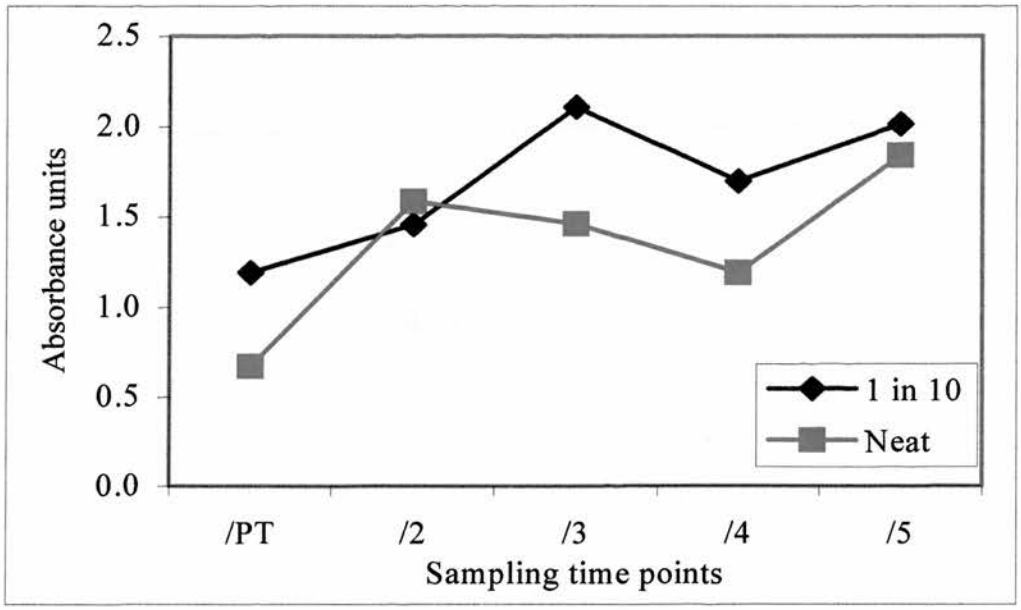
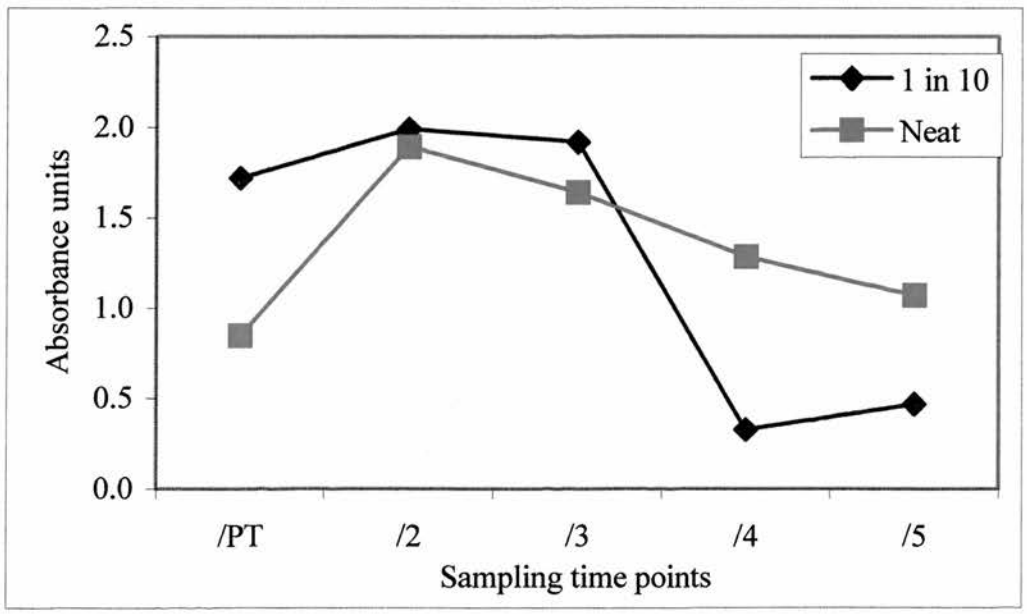


Figure 4.4.3b – IFN-γ series (patient 2)



Figures 4.4.3a and 4.4.3b show the RT-PCR ELISA profile for IFN-γ in two patients using neat and 1 in 10 dilution of cDNA (as labelled within each figure).

Figure 4.4.4a – IL-10 series (patient 1)

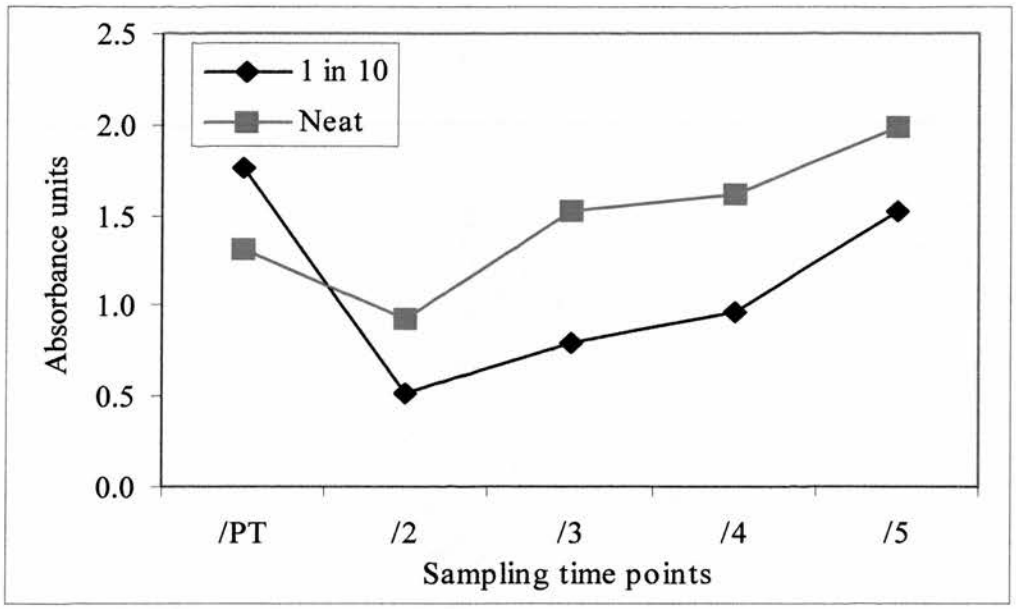
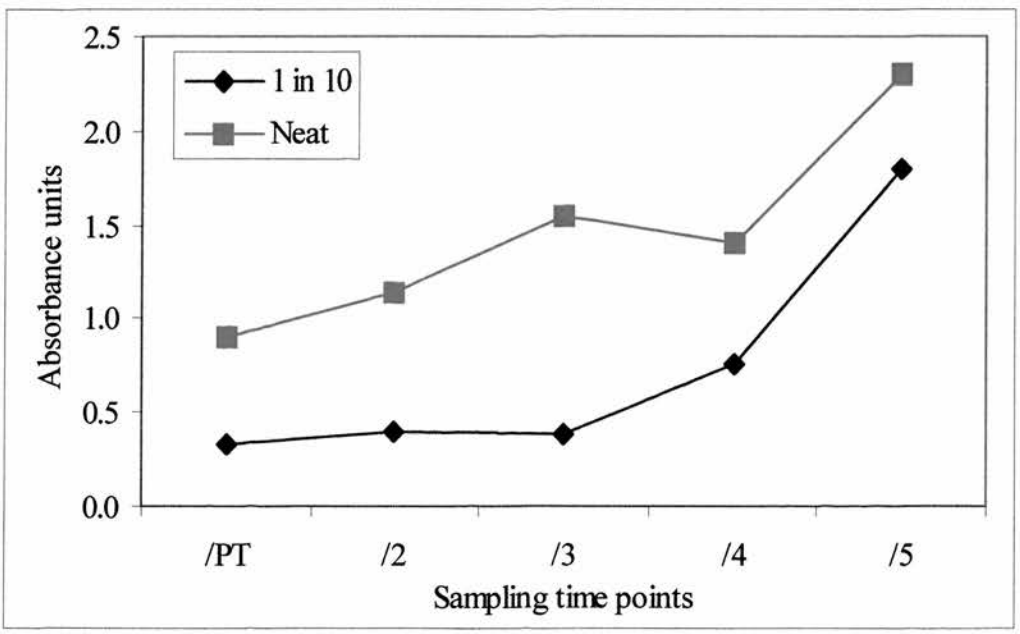


Figure 4.4.4b – IL-10 series (patient 2)



Figures 4.4.4a and 4.4.4b show the RT-PCR ELISA profile for IL-10 in two patients using neat and 1 in 10 dilution of cDNA (as labelled within each figure).

Discussion

Overall, the patterns of changes for all four cytokines in each patient series when neat concentrations of cDNAs were used were quite similar to the patterns seen for the same patient series when 1 in 10 dilutions of cDNAs were used instead.

The similarity in the patterns of changes was particularly true for IL-4 and IL-10, but more variability was seen for IL-2 and IFN-γ. Nevertheless, this variability is acceptable for biological data of this kind since the two experiments using different concentrations of cDNAs were not performed at the same, and therefore the PCR conditions for each experiment although the same in term of the protocol used, but would not be exactly the same in the practical sense.

It is therefore acceptable to use the cDNAs in neat concentrations or in diluted concentrations for assaying the gene expression levels in the project samples. Wherever possible, neat concentrations will be used (so that reasonable readings would be obtained for the lowly expressed cytokines like IL-2 and IL-5) but when there is a shortage of samples (usually in the post-anti-rejection samples), it was decided that a 1 in 5 dilution of cDNAs would be used instead. Obviously, for meaningful comparison of the levels of gene expression for each cytokine or CTL activation marker at the different time points for any given patient to be possible, all the samples for that patient would be assayed by RT-PCR ELISA using either neat or diluted cDNA.

Chapter 5: CLINICAL AND LABORATORY CHARACTERISATION OF PATIENT STUDY GROUP

This is a general results chapter summarizing the findings from different aspects of patients' data that were recorded in their project proformas (appendix 1). The first two sections describe the overall characteristics of the study patients and the apparent differences in these characteristics between the patients with and without early acute rejection episodes. The timing of the peripheral blood sampling from the patients recruited is set out in the next section, with a summary of the cell separation and RNA extraction data following this. The final two sections detail the biochemical renal function and cyclosporin profiles of the study patients and their subsequent clinical follow-up.

5.1 General patient details

With the full approval of the hospital's ethics committee (appendix 2), recruitment of patients for the study was conducted in two stages: between May 1996 and July 1996, and between October 1996 and May 1997. No pre-transplant selection criteria were imposed, with all patients being recruited whenever possible. Informed consent was obtained from each patient participating in the study (the consent form and patient information leaflet in appendices 3 and 4 respectively). The reason for the gap in the recruitment phase was to allow for optimisation of the laboratory protocols for subsequent molecular analyses and to obtain preliminary results, allowing the efficacy of the system to be evaluated before proceeding further.

A total of 58 patients were recruited from the transplant programme of the Wessex Renal and Transplant Unit at St Mary's Hospital, Portsmouth. Fifteen (25.9%) of the patients recruited were dropped from the study for the following reasons: 7 patients

(46.7%) had primary non-function of their renal allografts (as defined by the need for dialysis within the first week following transplantation, for reasons other than an elevated serum potassium), and 8 patients (53.3%) failed to have their transplants (6 patients because of positive patient-donor cytotoxic antibody cross-matches detected by complement-dependent cytotoxicity and/or flow cytometry and 2 patients who were deemed to be unfit for surgery by the anaesthetists). Hence the overall primary non-function rate for the study cohort was 14% (7 out of 50 transplants).

Of the 43 patients who were successfully recruited for the study, 26 (60.5%) were male. The median age of the patients was 43 years (range 22 to 70 years). Twenty-two (51.2%) of the patients were established on haemodialysis while 10 (23.3%) were on peritoneal dialysis. Eleven (25.6%) of the patients were pre-dialysis. The causes of end-stage renal failure of the study cohort are listed in table 5.1.1.

The majority of the recipients and donors were blood group A (table 5.1.2) in our study cohort, which is different from the national picture where group O is the commonest blood group [UKTSSA Transplant Activity 1996]. The CMV status of recipients and donors is summarised in table 5.1.3. The proportion of CMV positive recipients is in keeping with that in the general population of about 50% seroconversion by adulthood, although the number of CMV positive donors were slightly lower than would be expected. Overall, the majority of recipients (86%) received matched CMV status or CMV negative kidneys (table 5.1.4).

Twenty-nine (67.4%) patients received cadaveric kidneys while 14 (32.6%) had living-related renal transplants. Thirty-seven (86.0%) of the transplants were primary grafts, 5 (11.6%) were second grafts and only one (2.3%) was a fourth transplant. Twenty-five (58.1%) of the transplants were placed in the left iliac fossa.

Table 5.1.1 - Causes of end-stage renal failure

Causes of end-stage renal failure	No. of patients
Analgesic nephropathy	1
Chemotherapy for malignant testicular teratoma	1
Chronic pyelonephritis	3
Crescentic GN/Wegener's granulomatosis	1
Diabetes mellitus (insulin/non-insulin dependent)	3
Focal segmental glomerulosclerosis	2
Hereditary nephritis	1
Hypertension	3
IgA nephritis	4
Lupus nephritis	1
Membranous GN	1
Mesangio-IgA nephritis	1
Mesangio-capillary GN	2
Minimal change GN	1
Polycystic kidneys	4
Proliferative GN	1
Reflux nephropathy	4
Renal amyloidosis	1
Small kidneys	2
Unknown	6

Note: GN = glomerulonephritis

Table 5.1.2 - ABO blood groups of recipients and donors

	Group O	Group A	Group B	Group AB
Recipients	14 (32.5%)	26 (60.5%)	1 (2.3%)	2 (4.7%)
Donors	17 (39.5%)	24 (55.8%)	2 (4.7%)	0 (0%)

Table 5.1.3 - CMV status of recipients and donors

	CMV Positive	CMV Negative
Recipients	21 (48.8%)	22 (51.2%)
Donors	19 (44.2%)	24 (55.8%)

Table 5.1.4 – CMV matching between recipients and donors

	No. of transplants
Matched recipient/donor CMV status	29 (67.4%)
Recipient positive and donor negative	8 (18.6%)
Recipient negative and donor positive	6 (14.0%)

The HLA matching status of the transplants is detailed in tables 5.1.5 and 5.1.6. The importance of HLA matching for the HLA-DR locus was reflected by 97.7% of patients transplanted having at least one HLA-DR antigen matched with the donor, compared with only 81.4% and 67.4% of patients respectively with at least one HLA-A and HLA-B antigen match (table 5.1.5). The overall average total mis-match (i.e. the sum of mis-match at HLA-A, -B and -DR loci) per patient was 2.9 and 31 patients (72.1%) had 3 or less total HLA mis-matches.

As expected, the commonest pattern of HLA mis-match in our study cohort (16 patients, 37.2%) was a 1-A: 1-B: 1-DR mis-match and only 6 patients (14.0%) had a "full house" HLA match with their donors. Seven patients (16.3 %) received beneficially matched kidneys (where a beneficial match is defined as a 0:0:0, 1:0:0 or 0:1:0 pattern of HLA-A:-B:-DR mis-match). Based on the new UKTSSA favourable matching group formed by including the 1:1:0 pattern of HLA-A:-B:-DR mis-match with the beneficial match criteria [UKTSSA Transplant Activity, 1996], 9 patients (20.9%) in the study cohort received kidneys in this new group.

Table 5.1.5 - Tissue typing mis-match for each HLA antigen group

No. of mis-match	HLA-A	HLA-B	HLA-DR
Zero	8 (18.6%)	9 (20.9%)	11 (25.6%)
One	27 (62.8%)	20 (46.5%)	31 (72.1%)
Two	8 (18.6%)	14 (32.6%)	1 (2.3%)

Table 5.1.6 - Patterns of HLA mis-match

Pattern of A : B : DR mis-match (MM)	No. of transplants
0 : 0 : 0 (MM=0)	6 (14.0%)
1 : 0 : 0 (MM=1)	1 (2.3%)
1 : 1 : 0 (MM=2)	2 (4.7%)
1 : 0 : 1 (MM=2)	1 (2.3%)
1 : 2 : 0 (MM=3)	2 (4.7%)
0 : 2 : 1 (MM=3)	2 (4.7%)
1 : 1 : 1 (MM=3)	16 (37.2%)
2 : 0 : 1 (MM=3)	1 (2.3%)
1 : 2 : 1 (MM=4)	5 (11.6%)
2 : 1 : 1 (MM=4)	2 (4.7%)
2 : 2 : 1 (MM=5)	4 (9.3%)
2 : 2 : 2 (MM=6)	1 (2.3%)

5.2 Patients with early acute rejection

Of the 43 patients in the study, 15 patients (34.9%) had experienced early acute rejection (defined as acute cellular and/or vascular rejection occurring within the first 6 weeks following transplantation), with 14 (93.3%) of these patients experiencing their rejection episode within 3 weeks of their transplantation (of whom 10 (66.7%) occurred in the first 2 weeks). All diagnoses of acute allograft rejection were made by a combination of clinical and laboratory criteria of graft dysfunction, and confirmed histologically by graft biopsy.

The demographic characteristics of the two groups of patients within the study cohort were statistically comparable. The details of these demographic characteristics (age, sex distribution, type of transplant, transplant number, mode of pre-transplant renal replacement therapy, recipient and donor blood groups, recipient and donor CMV status and matching, and pattern and number of HLA mis-match) in patients experiencing early rejection and those who did not are shown in tables 5.2.1 to 5.2.6 respectively. No significant differences between the two groups (table 5.2.7) were found for any of these factors at the 5% level.

Table 5.2.1 – Age, sex distribution, proportion of cadaveric and primary transplants in patients with/without early acute rejections

Early acute rejections?	**Yes (n=15)**	**No (n=28)**
Median age of recipients (range)	49 years (26 - 59)	36 years (22-70)
Male recipients	7 (46.7%)	19 (67.9%)
Cadaveric transplants	12 (80.0%)	17 (60.7%)
Primary transplants	14 (93.3%)	23 (82.1%)

Table 5.2.2 – Methods of renal replacement therapy in patients with/without early acute rejections

Early acute rejections?	**Yes (n=15)**	**No (n=28)**
Haemodialysis	6 (40.0%)	16 (57.1%)
Peritoneal dialysis	5 (33.3%)	5 (17.9%)
None (i.e. pre-dialysis)	4 (26.7%)	7 (25.0%)

Table 5.2.3 – Donors and recipients blood groups in patients with/without early acute rejections

	Early acute rejections?			
	Yes (n=15)		**No (n=28)**	
Blood groups	**Donor**	**Recipients**	**Donor**	**Recipients**
O	7 (46.7%)	7 (46.7%)	10 (35.7%)	7 (25.0%)
A	7 (46.7%)	7 (46.7%)	17 (60.7%)	19 (67.9%)
B	1 (6.7%)	1 (6.7%)	1 (3.6%)	0
AB	0	0	0	2 (7.1%)

Table 5.2.4 – CMV matching in patients with/without early acute rejections

Early acute rejections?	**Yes (n=15)**	**No (n=28)**
Matched recipient/donor CMV status	12 (80.0%)	17 (60.7%)
Recipient positive and donor negative	2 (13.3%)	6 (21.4%)
Recipient negative and donor positive	1 (6.7%)	5 (17.9%)

Table 5.2.5 - Pattern of HLA mis-match in patients with/without early acute rejections

Pattern of A : B : DR mis-match (MM)	**Early acute rejections?**	
	Yes (n=15)	**No (n=28)**
0 : 0 : 0 (MM=0)	0	6 (21.4%)
1 : 0 : 0 (MM=1)	0	1 (3.6%)
1 : 1 : 0 (MM=2)	0	2 (7.1%)
1 : 0 : 1 (MM=2)	0	1 (3.6%)
1 : 2 : 0 (MM=3)	1 (6.7%)	1 (3.6%)
0 : 2 : 1 (MM=3)	0	2 (7.1%)
1 : 1 : 1 (MM=3)	8 (53.3%)	8 (28.6%)
2 : 0 : 1 (MM=3)	0	1 (3.6%)
1 : 2 : 1 (MM=4)	2 (13.3%)	3 (10.7%)
2 : 1 : 1 (MM=4)	2 (13.3%)	0
2 : 2 : 1 (MM=5)	1 (6.7%)	3 (10.7%)
2 : 2 : 2 (MM=6)	1 (6.7%)	0

Although the total HLA mis-match was greater in patients experiencing early acute rejection with an average total mis-match per patient of 3.7 (2.5 for patients not experiencing early acute rejections), this difference was not statistically significant (table 5.2.7). Nonetheless, it is interesting to observe that patients experiencing early acute rejections have less zero mis-match at all the three HLA loci (table 5.2.6) and only 60.0 % had 3 or less total HLA mis-match per patient (compared to the 78.6% of patients without early acute rejection episodes who had 3 or less total HLA mis-match).

Table 5.2.6 - HLA mis-match (MM) in patients with/without early acute rejections

	Early acute rejections?					
	Yes (n=15)			No (n=28)		
No. of mis-match	A	B	DR	A	B	DR
Zero	0	0	1 (6.7%)	8 (28.6%)	9 (32.1%)	10 (35.7%)
One	11 (73.3%)	10 (66.7%)	13 (86.7%)	16 (57.1%)	10 (35.7%)	18 (64.3%)
Two	4 (26.7%)	5 (33.3%)	1 (6.7%)	4 (14.3%)	9 (32.1%)	0

Of the 15 patients who experienced early acute rejection, 9 had histological evidence of acute cellular rejection only, while the other 6 patients had acute vascular rejection in addition to acute cellular rejection. All rejection episodes were treated in the first instance with a three-day course of methylprednisolone pulses (half a gram per day), although 3 patients received an extra dose and 2 patients had a second course of methylprednisolone to successfully treat their rejection episode.

Of the 6 patients with histological evidence of acute vascular rejection as well as acute cellular rejection, biological anti-rejection agents were used in 5 patients (4 with polyclonal antibodies and 1 with monoclonal antibodies). The remaining patient with vascular and cellular rejection had concomitant sepsis from transplant urinary leak and was therefore unable to tolerate further immunosuppression with biological agents to rescue his rejecting renal allograft. One other patient with acute cellular rejection only who failed to respond to methylprednisolone pulses was also given biological therapy with a polyclonal agent. Between 3 and 5 doses of each biological agent were given over a 10-day period depending on the degree of additional immunosuppression achieved with that agent as assessed by the daily FACS CD3 counts (the agent was given when the total CD3 count was greater than

50,000 per ml). The polyclonal antibodies used were anti-lymphocyte globulin, Lymphoglobuline® [Pasteur Merieux, France] or anti-thymocyte globulin, Thymoglobuline® [Pasteur Merieux, France] and the monoclonal antibodies used was Orthoclone OKT3® [Janssen-Cilag, Switzerland].

Table 5.2.7 – Statistical analysis of differences between patients with/without early acute rejections

	Statistical tests	**Pearson χ^2**	**df**	**p-value* (Fisher's exact test)**
Age distribution	*t* test	-	-	0.125
Sex distribution	χ^2 test	1.835	1	0.176 (0.206)
Cadaveric/living-related	χ^2 test	1.654	1	0.198 (0.308)
Previous transplants	χ^2 test	1.165	2	0.558
Renal replacement therapy	χ^2 test	1.578	2	0.454
Recipient blood group	χ^2 test	5.072	3	0.167
Donor blood group	χ^2 test	0.843	2	0.656
CMV matching	χ^2 test	1.759	2	0.415
Total HLA mis-match	χ^2 test	9.510	6	0.147

Notes: df = degrees of freedom; χ^2 = Chi squared
* 2-tailed p-value; Fisher's exact test applicable in 2X2 tables only

5.3 Timing of peripheral blood sampling

A total of 237 peripheral blood samples were taken from the 43 patients in the study. For the patients who did not experience any early acute rejection episode, 5 samples of peripheral blood per patient were taken within six weeks of their transplantation, while for those who experienced early acute rejections, a median of 6 samples (range 5 to 8) of peripheral blood per patient were taken.

The details of the timing of peripheral blood sampling from all patients without early acute rejection are summarised in table 5.3.1. For the patients with early acute rejection episodes, the same blood sampling schedule as for the patients without early acute rejection episodes was followed up to the time when acute allograft rejection was diagnosed and confirmed (table 5.3.2a). The time schedule for additional peripheral blood sampling prior to commencing anti-rejection therapy and following anti-rejection therapy in order to study the effect of acute allograft rejection and anti-rejection therapy on changes in gene expression levels is shown in table 5.3.2b. The medians and ranges in all the tables in this section refer to the number of days pre-transplant or post-transplant as indicated by the sampling time points. The day of transplantation was taken as day zero.

Table 5.3.1 – Protocol blood sampling time points (non-rejectors)

Sampling time points	**No. of patients with samples**	**Median (days)**	**Range (days)**
Pre-transplant	28	0	0 – 14*
Early post-transplant	28	2	2 - 4
1 week post-transplant	28	6	5 - 8
2 weeks post-transplant	28	12	10 - 14
4 weeks post-transplant	28	29	27 - 43

*days before transplantation

Most of the patients had their pre-transplant bloods sampled within 24 hours prior to their kidney transplantation. The 6 patients who had their pre-transplant bloods sampled between 2 and 14 days prior to their transplants were all living related transplants and their bloods were sampled earlier than the rest either because their transplants were postponed for medical reasons, or because it was convenient to take their pre-transplant blood at the time when they came into the unit for their T and B cell serological and FACS crossmatch.

Table 5.3.2a – Protocol blood sampling time points (early rejectors)

Sampling time points	No. of patients with samples	Median (days)	Range (days)
Pre-transplant	15	0	0 - 2
Early post-transplant	15*	2	2 - 4
1 week post-transplant	13	6	5 - 7
2 weeks post-transplant	8	12	10 - 14
4 weeks post-transplant	0	-	-

* see text for explanation

Table 5.3.2b – Additional blood sampling time points (early rejectors)

Sampling time points	No. of patients with samples	Median (days)	Range (days)
Pre-anti-rejection therapy	14*	1	0 - 5
Early post-anti-rejection therapy	15	2	1 – 5
1 week post-anti-rejection therapy	13*	7	6 – 10
4 weeks post-anti-rejection therapy	9*	28	18 - 36

* see text for explanation

For patients experiencing early acute rejection episodes, the early post-transplant samples were the same as the pre-anti-rejection therapy samples in two patients (table 5.3.2a).

Missing samples

There was no opportunity to take a sample prior to commencing anti-rejection therapy for one patient with early rejection as she was admitted from the outpatient clinic and given anti-rejection therapy before I could see her (table 5.3.2b). Unfortunately the same patient did not have the sample taken one week following the completion of her anti-rejection therapy due to logistical reasons. Another patient

also did not have the one week post-anti-rejection therapy sample taken because he had graft nephrectomy by then.

The 4 weeks post-anti-rejection therapy samples were only done on the last 9 patients who experienced early acute rejections because this time point was not in our original additional blood sampling schedule, it was only added to the protocol after preliminary data from the first 6 patients who had experienced early acute rejection suggested that it would be useful to obtain a further sample from these patients so that the immunological status when the rejection process has been fully overcome may be determined.

5.4 Cell separation and RNA extraction

The median volume of blood taken at each sampling time point (excluding the volume of citrate in the vacutainers) was 15.5 ml (range 8.1 - 19.0). The details of the cell separation results for the study cohort are set out in the appendix 6 and a summary of the results is shown in table 5.4.1.

The median interval between the addition of RNAzol B to the cell pellet at the end of the cell separation step and the extraction of the total RNA from the RNAzol B homogenates was 2 days (range 1 to 17 days). The details of the RNA extraction results are set out in appendix 7. A summary of the results is shown in table 5.4.2. The lower total mononuclear cells (MNC) available for total RNA extraction was discounted by the small volume of MNC suspension removed for cell count by the Coulter counter. Overall, the total RNA extracted by the chosen method yielded a reasonable quantity of total RNA with high purity (free of DNA and proteins) and has a 260/280 absorbance ratio higher than 1.9 (according to the maker of RNAzol B).

Table 5.4.1 - Summary of cell separation results

Cell counts		Median	5th Percentile	95th Percentile
Before cell separation	LY (x10^6/ml)	1.3	0.3	2.9
	MO (x10^6/ml)	0.6	0.2	1.3
	% Monocytes	32.8	12.5	60.0
	MNC (x 10^6)	28.8	10.8	57.7
After cell separation	MNC (x 10^6)	12.0	3.0	29.7
	% cell loss	58.2	37.5	79.7

Note: LY= lymphocyte count; MO=monocyte count; MNC=mononuclear cell count

Table 5.4.2 - Summary of the RNA extraction results

	Median	5th Percentile	95th Percentile
MNC (x 10^6)	11.6	2.9	28.7
Purity (260/280 ratio)	2.0	1.9	2.1
Total RNA (μg)	11.8	2.9	37.3
Total RNA/10^6 cells (μg)	1.2	0.4	2.4

Note: MNC=mononuclear cells

5.5 Biochemical renal function profiles & cyclosporin levels

For clarity, table 5.5.1 lists the blood sampling time points numerically as used in annotating the x-axes of all the graphs in this section.

In all the graphs, the open circles (o) denote outliers (defined as cases with values between 1.5 and 3 box lengths from the upper or lower edge of each box plot), while the asterisks (*) denote the extremes (defined as cases with values more than 3 box

lengths from the upper or lower edge of each box plot). The box length represents the interquartile range. Both the outliers and extremes data are labelled with the case numbers of the patients as they were entered into the SPSS database. N refers to the number of results available.

Table 5.5.1 – Numbering of time points used in the graphs

Numbering	Time points
1	Pre-transplant
2	Early post-transplant
3	1 week post-transplant
4	2 weeks post-transplant
5	4 weeks post-transplant
6	Pre-anti-rejection therapy
7	Early post-anti-rejection therapy
8	1 week post-anti-rejection therapy
9	4 Weeks post-anti-rejection therapy
10	3 months post-transplant
11	6 months post-transplant
12	12 months post-transplant

The overall biochemical renal function profiles of the patients with and without early acute rejection episodes are shown in figures 5.5.1 to 5.5.4. No formal statistical test was applied to any of these data as the trends of changes are obvious and do not require statistical tests to demonstrate them.

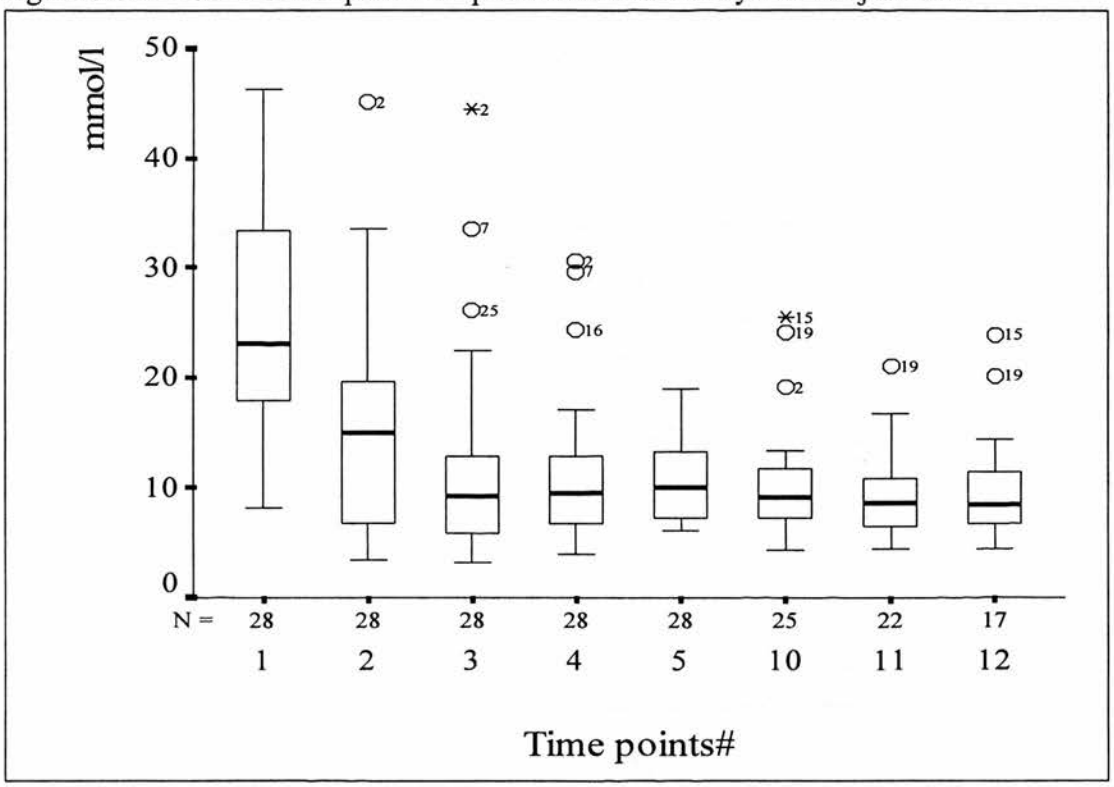
Notice that the majority of the acute rejection episodes had been successfully treated as seen by the sizeable fall in the mean serum urea and creatinine of the patients by the end of the first week following anti-rejection therapy (time point 8) in figures 5.5.2 and 5.5.4 respectively. The mean fall in serum urea and creatinine between pre-anti-rejection therapy (time point 6) and 1 week following anti-rejection therapy (time point 8) were 18.9% and 21.6% respectively, with nearly 70% of patients (9 out of 13 available comparisons) recording a fall of more than 10%.

Within the subgroup of patients who did not experience early acute rejections, the evidently superior quality of living-related kidneys as compared to cadaveric kidneys is demonstrated by an earlier and greater fall in serum urea and creatinine from the pre-transplant level following renal transplantation in this subgroup of patients (figures 5.5.5 to 5.5.8). This subgroup analysis comparing the performance of cadaveric with living-related kidneys was not applied to the patients who experienced early acute rejections because the numbers in the two groups would be too small for the comparison to be meaningful.

The cyclosporin A profiles of the patients without and with early acute rejections are set out in figures 5.5.9 to 5.5.10 respectively. The steady rise in plasma cyclosporin A levels in the first two weeks following transplantation in both groups of patients corresponded with the initial loading up of cyclosporin A during that period. Interestingly, although the cyclosporin A level in the early post-transplant period was similar in the two groups of patients, it was clearly higher in patients who had early acute rejections at 2 and 4 weeks post-transplant, suggesting that at least in this group of patients most of the early acute rejections episodes were not due to inadequate immunosuppression.

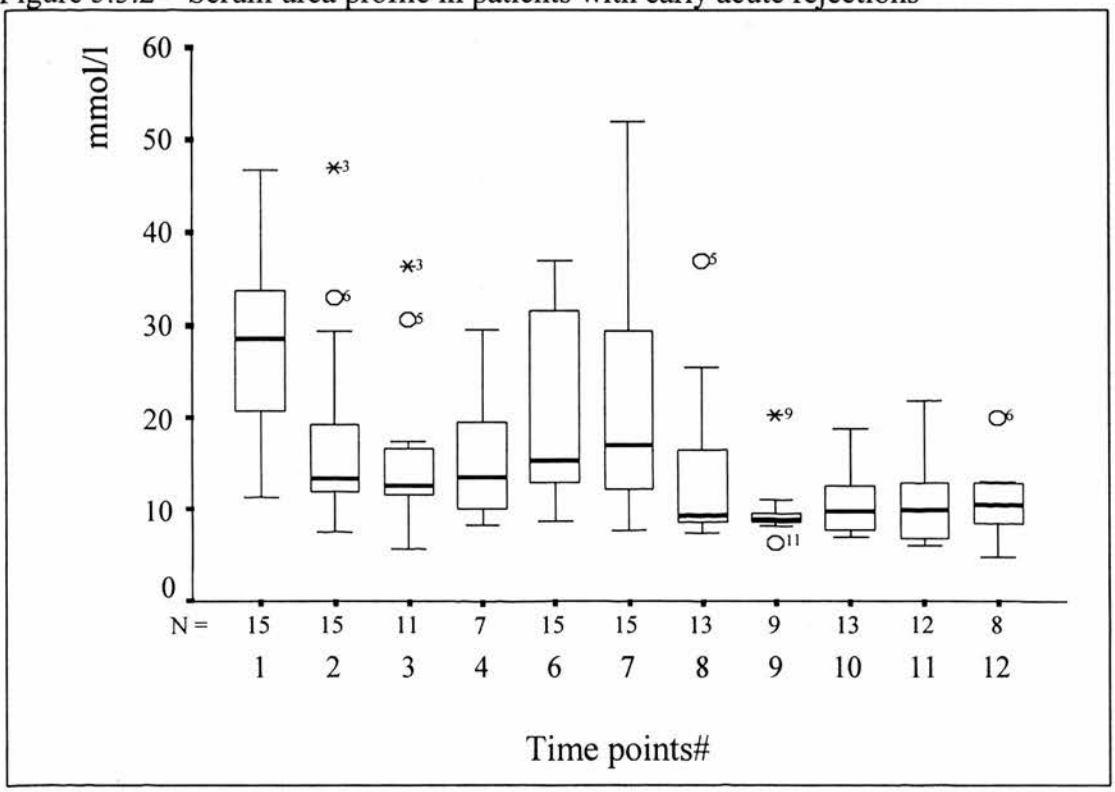
In the medium term follow-up (3 to 12 months post-transplant), the mean plasma cyclosporin A level in both groups of patients were similar, the mean level settling down to the target level of between 100 and 200 ng/ml from 3 months post-transplant onwards.

Figure 5.5.1 – Serum urea profile in patients without early acute rejections



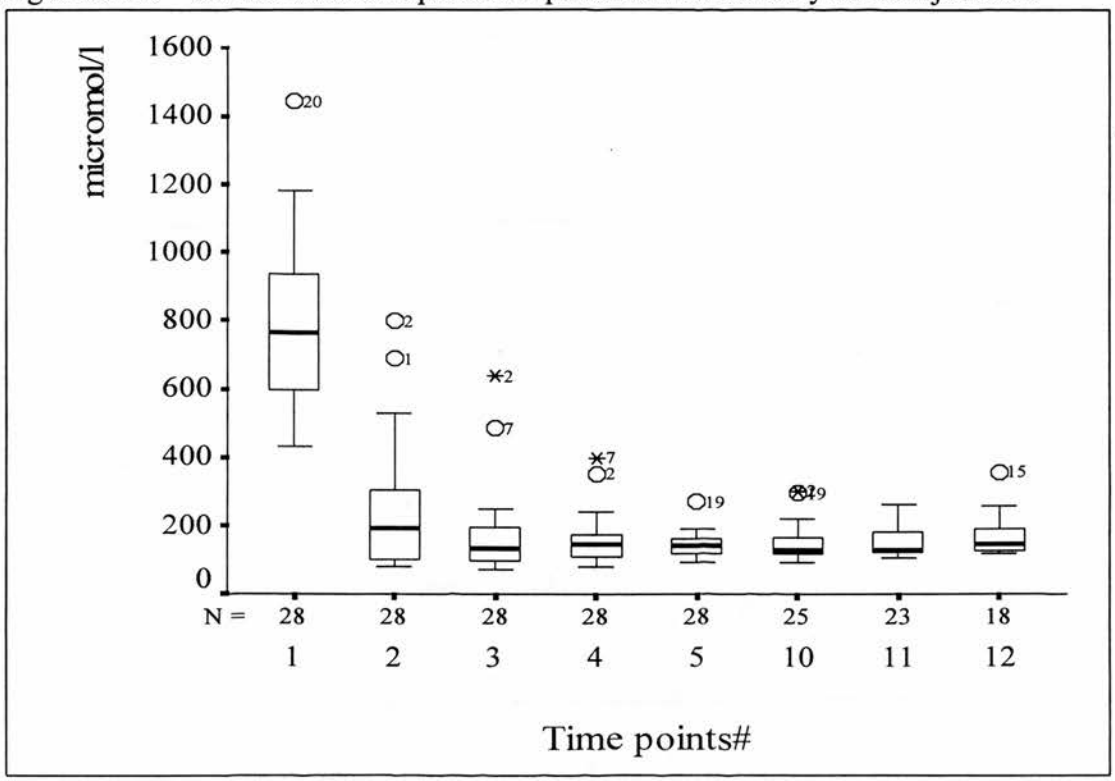
refer to table 5.5.1 for the numbering of time points

Figure 5.5.2 – Serum urea profile in patients with early acute rejections



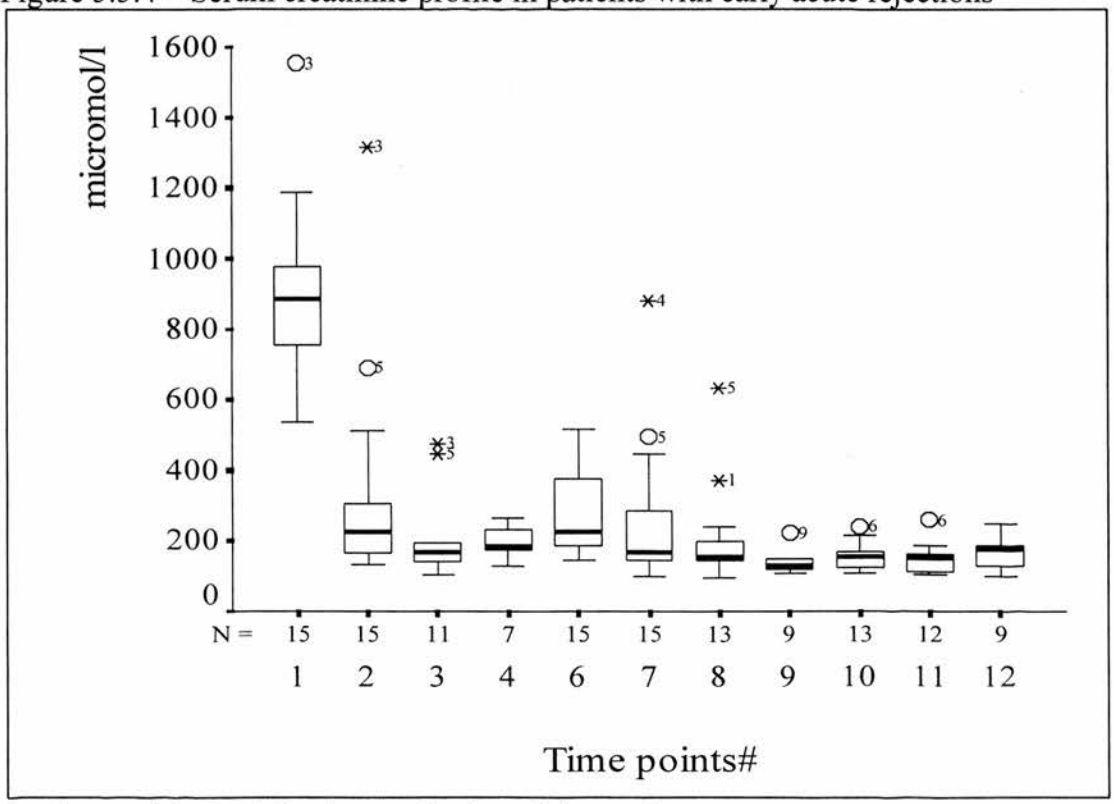
refer to table 5.5.1 for the numbering of time points

Figure 5.5.3 – Serum creatinine profile in patients without early acute rejections



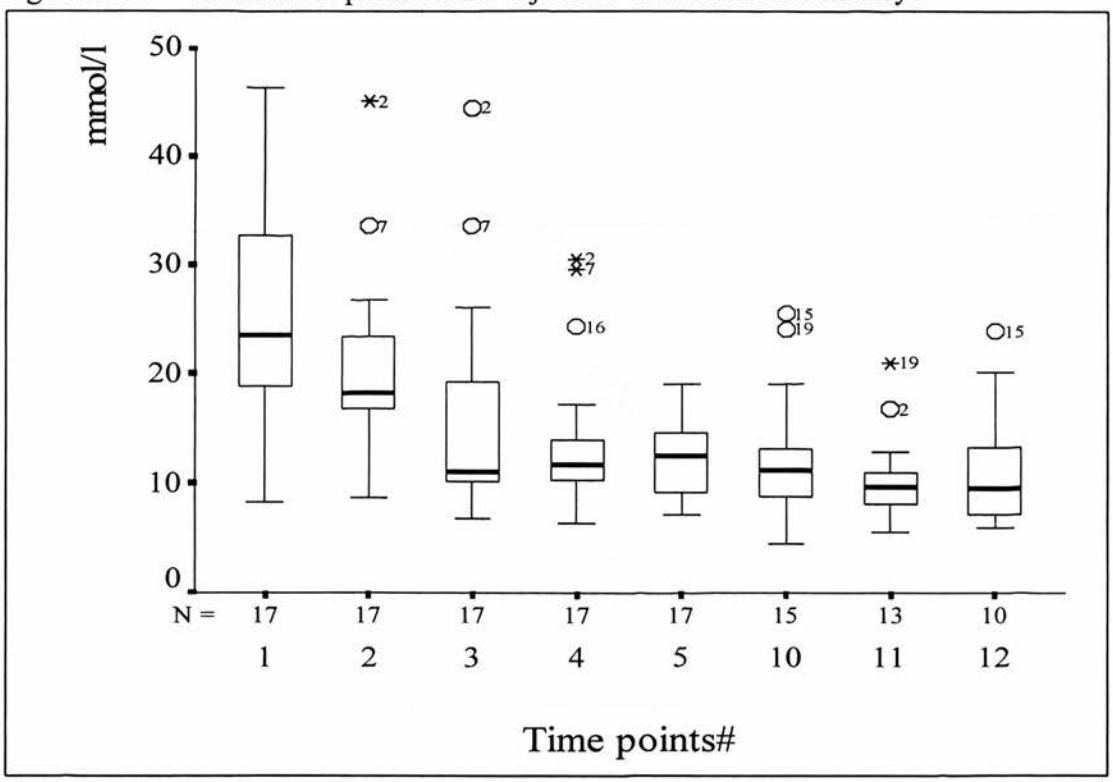
refer to table 5.5.1 for the numbering of time points

Figure 5.5.4 – Serum creatinine profile in patients with early acute rejections



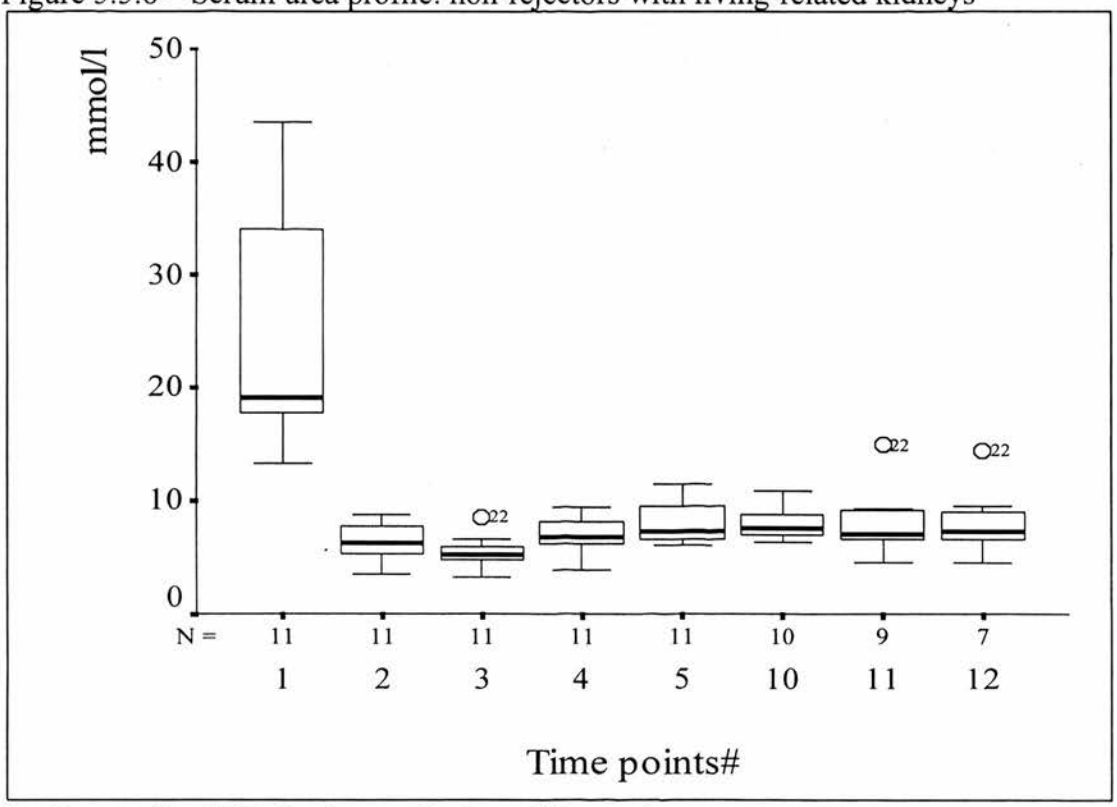
refer to table 5.5.1 for the numbering of time points

Figure 5.5.5 – Serum urea profile: non-rejectors with cadaveric kidneys



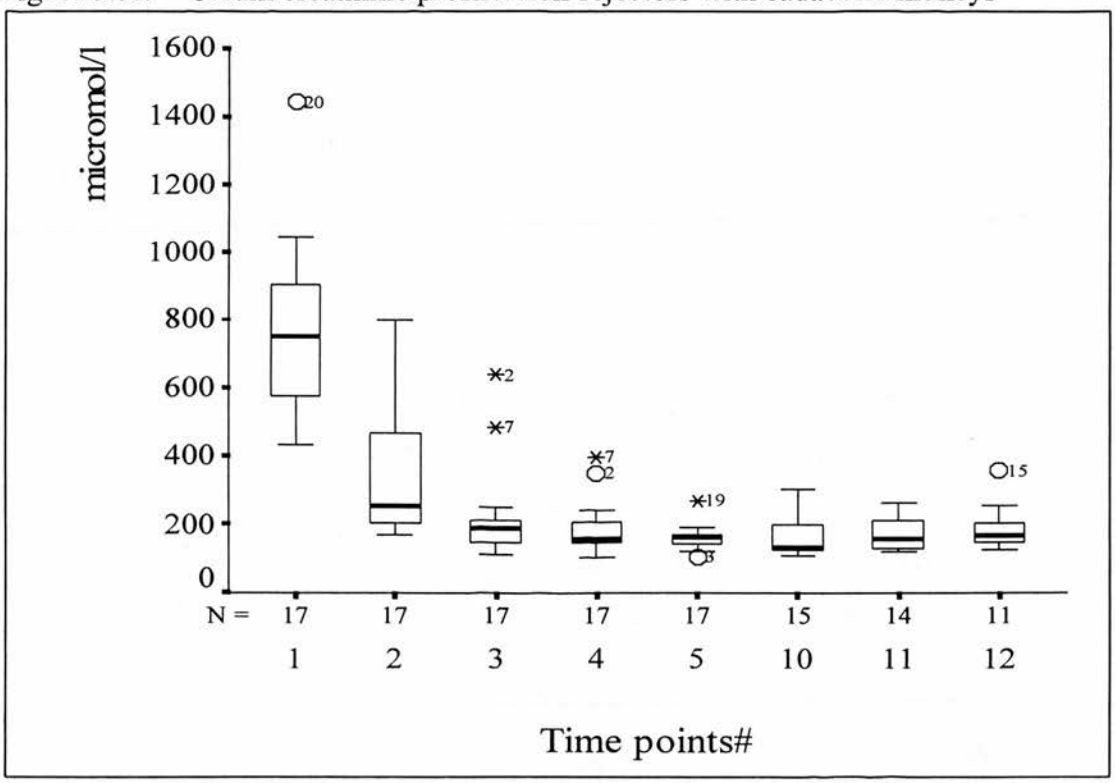
refer to table 5.5.1 for the numbering of time points

Figure 5.5.6 – Serum urea profile: non-rejectors with living-related kidneys



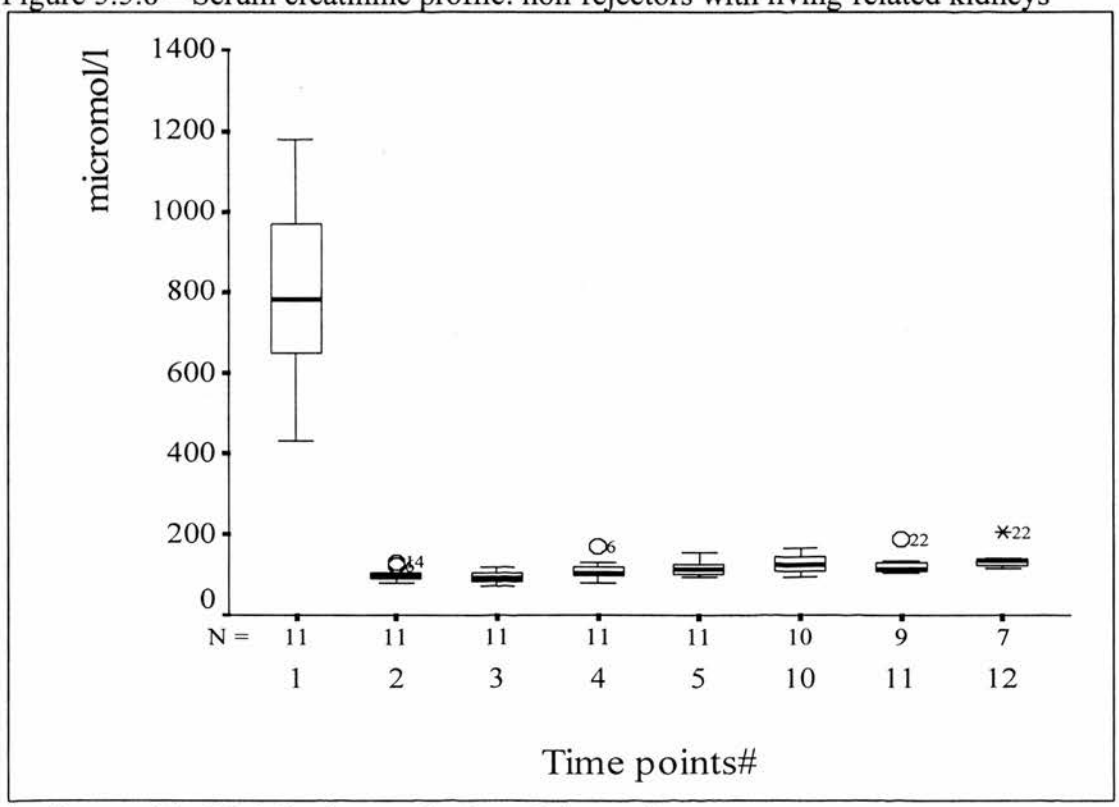
refer to table 5.5.1 for the numbering of time points

Figure 5.5.7 – Serum creatinine profile: non-rejectors with cadaveric kidneys



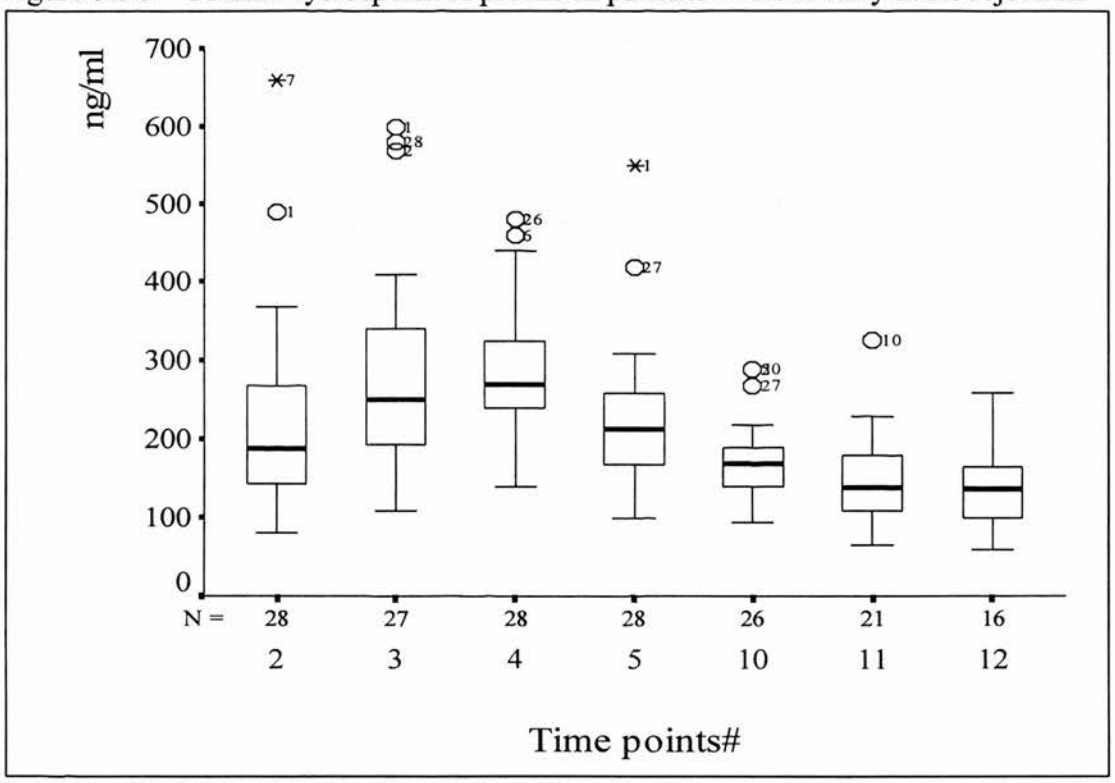
refer to table 5.5.1 for the numbering of time points

Figure 5.5.8 – Serum creatinine profile: non-rejectors with living-related kidneys



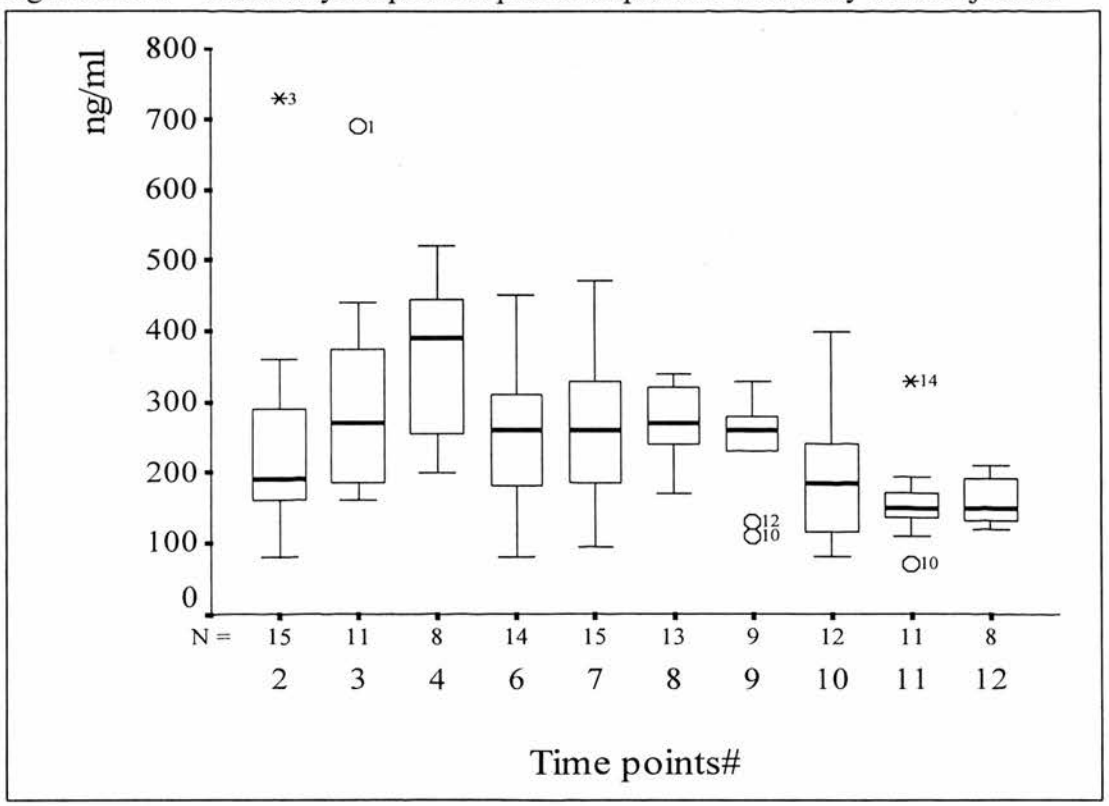
refer to table 5.5.1 for the numbering of time points

Figure 5.5.9 – Plasma cyclosporin A profile in patients without early acute rejection



refer to table 5.5.1 for the numbering of time points

Figure 5.5.10 – Plasma cyclosporin A profile in patients with early acute rejection



refer to table 5.5.1 for the numbering of time points

5.6 Follow-up clinical data

5.6.1 Medium term follow-up data

The clinical follow-up data detailed in this section have been included for completeness. These data are intended to give a more complete clinical picture of what happened to the study cohort following transplantation, by detailing the subsequent clinical events experienced by the study patients.

Having excluded the 2 patients who had graft nephrectomies and therefore had no meaningful follow-up data, all the remaining study patients were followed up to 12 months following their renal transplantation whenever possible. All the medium term follow-up data were recorded at fixed time points of 3, 6 and 12 months post-transplant to allow a reasonable time-frame comparison of the follow-up data.

Of the 41 patients left to follow-up, there were 3 (7.3%) patients with missing data at 3-month follow-up, two of whom were discharged early to their health authority outside the district and another patient had returned to the USA following her living-related transplant. By the 6-month follow-up, the number of missing data had increased to 5 (12.2%) patients, caused again by patients being discharged to health authorities outside the district. For the 12-month mark, there were 14 (34.1%) patients with missing data, but this further increase in the number of missing follow-ups were due mainly to these patients being recruited in the last two months of the recruitment phase and thus their 12-month follow-up had not come up by the time I left my research post in March 1998.

5.6.2 Outcome of anti-rejection therapy, further rejection episodes and mortality

Of the 15 patients who experienced early acute rejections, 2 lost their grafts. The graft nephrectomies were performed at 3 and 11 weeks post-transplant, and the histology of both grafts did confirm acute cellular and vascular rejection, with the

presence of acute pyelonephritis being seen also in the graft from a patient who had urinary leak.

The other 13 patients responded well to the anti-rejection therapy. One patient who had an acute cellular and vascular rejection 2 days following his living-related transplant which was successfully rescued with a combination of pulse methylprednisolone and anti-thymocyte globulin therapy, had a late rejection episode suggested by a deterioration in renal function and a biopsy showing borderline cellular rejection with some degree of vascular rejection as well as evidence of chronic rejection 8 months post-transplant. He was given a further pulse of methylprednisolone therapy and was converted from azathioprine to mycophenolate mofetil as a second line rescue therapy.

Of the 28 patients who did not experience early acute rejection, 2 had a suspected acute rejection episode 3 months post-transplant. Both were treated with pulse methylprednisolone therapy even though their biopsies showed non-specific changes only. One patient with a borderline cellular rejection confirmed on biopsy at over 4 months post-transplant was treated with pulse methylprednisolone and another episode of biopsy-proven cellular rejection occurred a month later requiring rescue with tacrolimus. This patient was already commenced on mycophenolate mofetil immunosuppression for this second living-related transplant, having lost his first cadaveric kidney to acute rejection. Another patient with a progressive deterioration in graft function from about 6 months post-transplant was found to have evidence of chronic tubular damage on biopsy together with an element of cellular rejection, but this did not respond to pulse methylprednisolone therapy.

Overall, one patient died 9 months post-transplant. He was an insulin-dependent diabetic who suffered a stroke about 3 weeks following a cadaveric renal transplant. Although he recovered well from his stroke, he subsequently succumbed to the diabetic complications affecting his feet despite intensive surgical interventions, including amputations, and marked reductions in the level of his immunosuppression.

5.6.3 Changes in immunosuppressive therapy

The immunosuppressive regimens used by the patients are shown in table 5.6.1. The majority of patients remained on triple immunosuppressive regimens, with the standard triple regime of cyclosporin A, prednisolone and azathioprine being the commonest immunosuppressive regimen. The patient who was on cyclosporin A, prednisolone and mycophenolate mofetil (marked with * in table 5.6.1) was commenced on this regime from the outset as he had received a living-related second transplant, having lost his first cadaveric transplant to acute rejection. He was converted from cyclosporin A to tacrolimus by his 6-month follow-up following a late acute rejection episode. One patient was converted from cyclosporin A to tacrolimus following the successful rescue of her early acute rejection with a combination of pulse methylprednisolone and anti-thymocyte globulin (marked with ** in table 5.6.1). Another patient mentioned in the previous section was converted from azathioprine to mycophenolate mofetil as a second line rescue therapy following a further episode of acute rejection and so showed up as a change in immunosuppression at 12 month follow-up (marked with † in table 5.6.1).

The 3 patients who were only on cyclosporin A and prednisolone at their 3 month follow-up had their azathioprine stopped temporarily because of a low leucocyte count, two of them having been on intravenous ganciclovir for acute CMV disease shortly prior to their 3 month follow-up appointment. The two patients who were on dual therapy only at their 12 month follow-up had the third immunosuppressive stopped deliberately by their nephrologists, the prednisolone was stopped as part of their long term immunosuppressive policy while the azathioprine was stopped because of leucopaenia.

Table 5.6.1 – Immunosuppressive regimens

	Months post-transplant follow-up		
Immunosuppressive regimens used	**3**	**6**	**12**
Cyclosporin A, Prednisolone, Azathioprine	33	34	22
Cyclosporin A, Prednisolone, Mycophenolate mofetil	1*	0	1†
Tacrolimus, Prednisolone, Azathioprine	1**	1**	1**
Tacrolimus, Prednisolone, Mycophenolate mofetil	0	1*	1*
Cyclosporin A, Prednisolone	3	0	1
Cyclosporin A, Azathioprine	0	0	1

5.6.4 Infective complications in the post-transplant period

The early infective complications (those occurring during the patients' first inpatient stay following their renal transplant) whether confirmed by laboratory investigation or suspected clinically, are detailed in table 5.6.2. Although the numbers in each category are set against each post-transplant time point, they refer to the infective complications that were present either at or during the time period immediately preceding that stated post-transplant time point. The category "Chest" refers to the presence of clinical and radiological evidence of chest infection but not necessarily with positive sputum culture, while the category "Urine" refers to the presence of urinary tract infection confirmed with positive culture of pathogenic organisms from mid-stream specimens or catheter specimens of urine. The category "Others" lists the individual infective complications other than chest or urinary tract infections and "PUO" refers to patients having a temperature greater than 37.5 ^{0}C with no obvious cause. "N" denotes the total number of patients with available post-transplant data for analysis.

Table 5.6.2 – Early infective complications

Protocol time points	N	Source of infection			PUO
		Chest	Urine	Others	
Early post-transplant	43	2	0	0	6
1 week post-transplant	41	1	4	0	3
2 weeks post-transplant	37	1	1	1 Cold	2
4 weeks post-transplant	29	0	1	1 Dental abscess	0

The prospective evaluation of the number of early infective complications detailed in table 5.6.2 refers only to the infective complications recorded in the proforma during each protocol blood sampling time point. Since most of the blood sampling time points prior to and following anti-rejection therapy for the patients who experienced early acute rejections usually fall outside these protocol blood sampling time points, no data on infective complications were recorded in the proforma for these patients. However, a detailed retrospective review of the transplant flow charts of these patients did not reveal any additional early infective complications.

At the 3-month, 6-month and 12-month follow-ups, none of the patients had experienced any chest, urinary tract or other infections during these follow-up periods. The only infective complication during the late follow-up period was acute CMV disease occurring in 4 patients, presenting at between 6 and 11 weeks post-transplant and all were treated with intravenous ganciclovir for three weeks. Three of the patients had primary CMV disease, having been CMV negative prior to their transplantation and had received CMV positive kidneys, while the fourth patient's acute CMV disease was a reactivation of her previous CMV infection, as she was CMV positive prior to her transplant and had received a CMV negative kidney. Interestingly, all the 3 patients who had primary CMV disease did not experience any rejection episodes while the patient who had reactivated CMV disease had experienced severe acute cellular and vascular rejection requiring two courses of

pulse methylprednisolone therapy as well and biological anti-rejection agent to rescue her kidney.

5.7 Discussion

The study group characterisation, sampling strategy and transplant outcome data presented in this chapter establish the background for the main body of this thesis, namely, the molecular analyses of cytokines and CTL activation markers gene expression of peripheral T lymphocytes in patients following renal transplantation, which is described in the following chapter. More specifically, this analysis showed no significant differences between those patients who did experience early acute rejection and those who did not experience early acute rejection of their renal allografts.

The proportion of living-related transplants in the study cohorts was considerably higher than the Wessex Renal and Transplant Unit's living-related transplant rate of around 15%. This skew towards living-related transplants in the study cohorts was inevitable because logistically it was much easier to recruit these patients than those receiving cadaveric kidneys since living-related transplants were elective operations, whereas cadaveric transplantations may take place outside of normal working hours and therefore the patients may be brought in for their transplants at times unsuitable for the laboratory work that accompanied the recruitment process. Apart from this, none of the other characteristics of the study patients were particularly unusual for renal transplant patients.

The statistical analysis of the patients' characteristics between those who had experienced early acute rejection and those who had not has clearly demonstrated that these two groups of patients were comparable. The medians and ranges of the timing of all the protocol peripheral blood sampling following transplantation were also very similar between the two groups. Therefore, any differences in the subsequent

molecular analyses between them cannot be accounted by any apparent differences between their patients' characteristics or the timings of their peripheral blood sampling.

The pattern of changes in the plasma levels of cyclosporin A in the post-transplant period is as one would expect from a policy of regular optimisation of the dose of cyclosporin A based on a twice weekly plasma cyclosporin A assay. The relative ease of achievement of the target range of plasma cyclosporin A in the patients is a reflection of the much improved absorption kinetics of the new microemulsion formulation of cyclosporin A, Neoral, resulting in a better correlation of plasma level of the drug with the oral dose.

The biochemical renal function profiles of the patients in the study have provided the documentary evidence of the dramatic impact of renal transplantation, as well as the impact of the acute rejection process and its treatment on these parameters. The marked superiority in the quality of living-related kidneys over cadaveric kidneys has been clearly demonstrated with the biochemical renal function profiles in the two groups.

The medium term follow-up data has completed the clinical picture of the study cohorts. Overall, graft loss in the study cohort was small, with 2 lost to acute rejections and one from patient death. The number of immunological events occurring after the study period was also small, with 3 patients overall with histological evidence of further acute cellular/vascular rejection or chronic rejection, although there were 2 other patients with suspected but unconfirmed acute rejection episodes.

Chapter 6: SEQUENTIAL GENE EXPRESSION PROFILES OF PERIPHERAL T CELLS BASED ON RT-PCR ELISA MONITORING

This chapter sets out the analyses of the molecular results of the project, namely the semi-quantitative RT-PCR ELISA data relating to the sequential changes in the level of cytokine and CTL activation marker gene expression in the early post renal transplant period. The RT-PCR ELISA data have been analysed in three ways, and each is set out in separate sections.

The first section looks at the profile of changes for each cytokine/CTL activation marker studied in the project in the two groups of patients, those who did not experience early acute rejection episodes (henceforth described as "non-rejectors") and those who did (henceforth described as "rejectors"). For the non-rejectors, data from all 5 protocol sampling time points were included in the analysis, while for the rejectors, the additional sampling time points around the acute rejection episode (i.e. the time point prior to commencing anti-rejection therapy and the time points following completion of the anti-rejection therapy) were analysed together with the first 2 protocol sampling time points for comparison. The next section looks at the cytokine and CTL activation marker gene expression profiles over the protocol sampling time points prior to the acute rejection episode in the rejectors and compares them with the profiles over the same protocol sampling time points in the non-rejectors. The final section compares the data at each protocol sampling time prior to the acute rejection episode in the rejectors with the data at the same time points in the non-rejectors.

Statistical analyses applied in the graphs

The RT-PCR ELISA data at all sampling time points (protocol sampling time points and the additional sampling time points) were compared using the non-parametric Wilcoxon signed rank sum test for matched pairs in the following analyses:

(i) Sequential analysis: the data (percentage change or ratio) at one time point was compared with the data at the time point immediately preceding it.

(ii) Pre-transplant baseline analysis: the data at all post-transplant time points were compared with the baseline pre-transplant time point.

(iii) Pre-rejection baseline analysis: for the rejectors, in addition to the first two analyses, the data in all the time points following anti-rejection therapy were compared with a second baseline, the pre-anti-rejection therapy time point.

Annotation of the graphs

The results of all the RT-PCR ELISA data are expressed in the standard box and whiskers plots. In all the graphs shown in this chapter, the outliers and extremes data are not shown for the sake of clarity, although it is emphasized that all data were included in the statistical analyses. The different sampling time points together with the number of samples at each time point are shown along the x-axis, while the y-axis gives the percentage change from the pre-transplant sample, which was taken as the baseline. All the raw data from the RT-PCR ELISA molecular analyses are shown in the appendices 8 to 11. A 2-tailed p value is quoted for all statistical analyses, and in all the graphs, only the p values obtained from the sequential analysis were quoted. The p values from the other two analyses are stated in the individual sections commenting on the profile of changes in the level of each cytokine/CTL activation marker in the study patient groups. Note also that both in the text and in the figures, only significant p values are quoted, otherwise the abbreviation "NS" for not significant will be used to denote all p values greater than 0.05.

6.1 Analysis of cytokine and CTL activation marker gene expression profiles in patients with and without early acute rejections

In the following sub-sections, sequential changes in cytokine (IL-2, IFN-γ, IL-4, IL-10, IL-5 and IL-13) and CTL activation marker (GrB and FasL) gene expression are considered in turn, and expressed in terms of the percentage change in the amount of their corresponding PCR products (expressed numerically by the ELISA plate readings) for each gene transcript at each post-transplant time point compared with the pre-transplant baseline (set at 100%). The validity of using the changes in the ELISA plate readings as a measure of the changes in the level of gene expression of a given cytokine has been shown by the various preliminary experiments validating the molecular techniques used in the project as detailed in chapter 4. These changes are plotted in figures 6.1.1 to 6.1.8, with the graphs for the non-rejectors and the rejectors set out next to each other in graphs (a) and (b) respectively within each figure. In the rejectors, the only pre-rejection time points included in the analysis here were the pre-transplant baseline and the early post-transplant time points.

All 15 patients experiencing early acute rejection were analysed for all cytokines and CTL activation markers listed above. All 28 non-rejectors were analysed for IL-2, IFN-γ, IL-4 and IL-10. Twenty-seven out of 28 non-rejectors were analysed for IL-5, IL-13, GrB and fasL, the second phase of molecular analyses, because the early post-transplant sample from one patient was not available for analysis, it was decided that analysing the remaining samples from this patient with the missing early post-transplant sample would not be appropriate as data analysis would be difficult because of the missing sample.

Overall from the ELISA plate readings, IL-2 and IL-5 have a general tendency towards a lower level of expression compared to the other cytokines or CTL activation marker at any time points and in both non-rejectors and rejectors (see the appendices 8 and 9 respectively for the raw ELISA plate readings).

6.1.1 IL-2 (figure 6.1.1)

In the non-rejectors, there were no significant differences in the levels of IL-2 gene expression not only sequentially from one time point to another, but also when all post-transplant time points were compared with the pre-transplant baseline.

In the rejectors, however, there was a significant fall in the level of IL-2 gene expression in the early post-transplant period ($p=0.023$). There were no further significant sequential changes in any of the subsequent time points. Nevertheless, this low level of IL-2 gene expression remained significantly lower than the pre-transplant baseline at the pre-anti-rejection therapy ($p=0.035$) and early post-anti-rejection therapy time points ($p=0.001$), returning to the pre-transplant baseline 1 week and 4 weeks following anti-rejection therapy (p=NS when these time points were compared with the pre-transplant baseline).

There were no significant differences in the level of IL-2 between all 3 time points following anti-rejection therapy and the pre-anti-rejection therapy time point.

6.1.2 IFN-γ (figure 6.1.2)

Like IL-2, no significant differences in the levels of IFN-γ gene expression were found in the non-rejectors both sequentially between all the time points and between all post-transplant time points and the pre-transplant baseline.

In the rejectors, as for IL-2, there was a significant fall in IFN-γ gene expression in the early post-transplant period ($p=0.003$), with no further significant sequential changes in the level of IFN-γ at all time points following this. However, unlike IL-2, the level of IFN-γ at the time of rejection (pre-anti-rejection therapy time point) was not significantly different from the pre-transplant baseline, but at the early post-anti-rejection therapy time point, IFN-γ level was again significantly below the pre-transplant baseline ($p=0.027$). IFN-γ level was not significantly different from the pre-transplant baseline level at 1 and 4 weeks following anti-rejection therapy.

Figure 6.1.1a

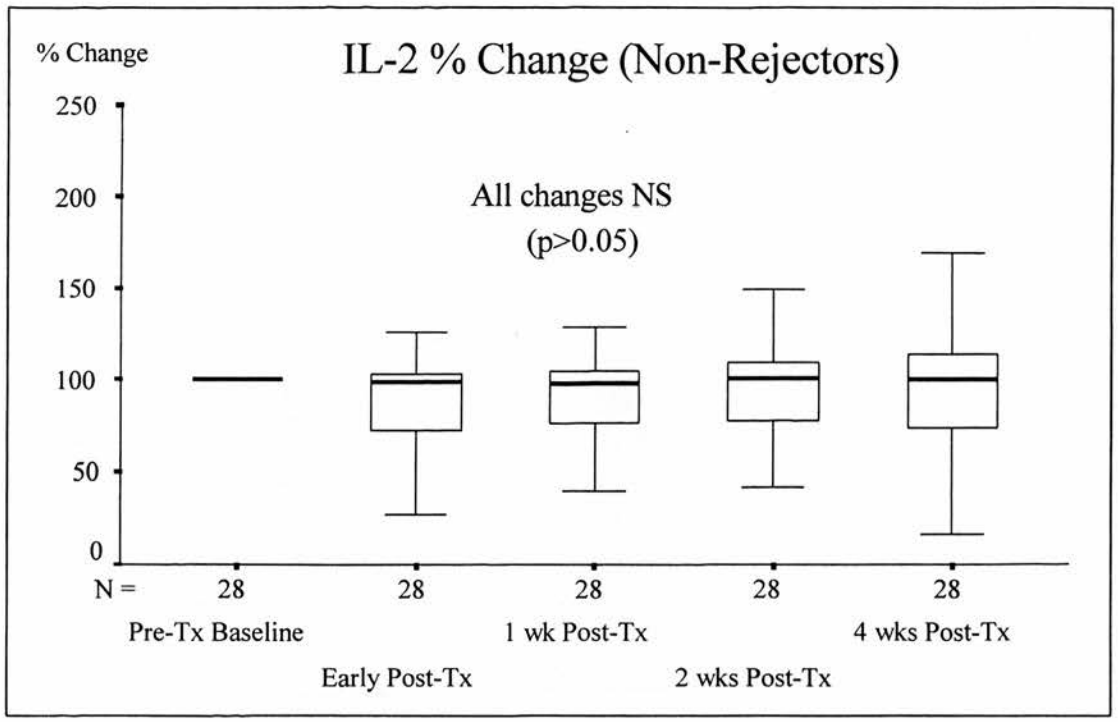


Figure 6.1.1b

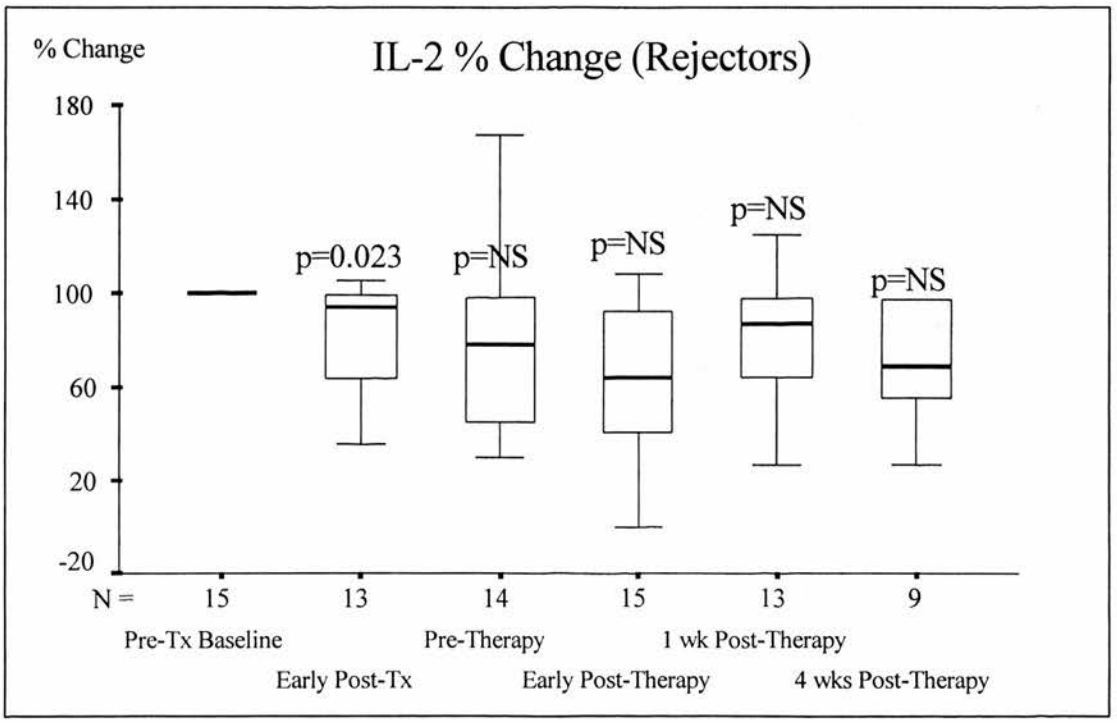


Figure 6.1.1 - Percentage change in IL-2 gene expression in non-rejectors and rejectors

Figure 6.1.2a

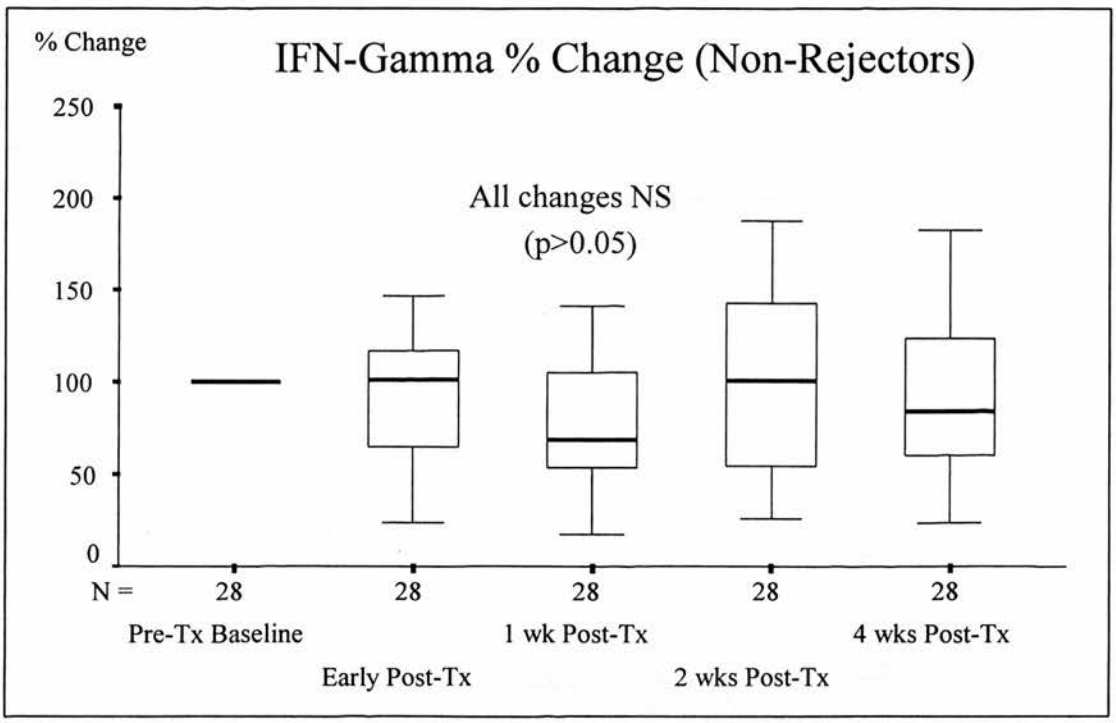


Figure 6.1.2b

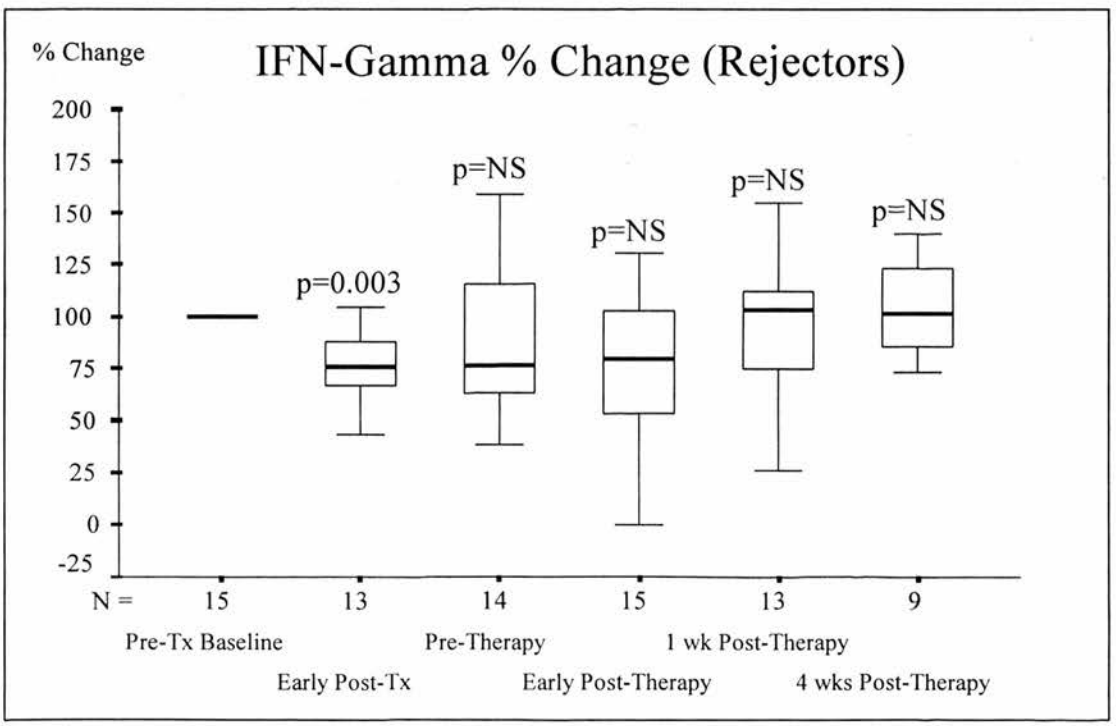


Figure 6.1.2 - Percentage change in IFN-γ gene expression in non-rejectors and rejectors

As for IL-2, there were no significant differences between the pre-anti-rejection therapy level of IFN-γ and all the post-anti-rejection therapy time points.

6.1.3 IL-4 (figure 6.1.3)

IL-4 gene expression in peripheral T cells showed the most sequential changes of all the cytokines and CTL activation markers studied. In the non-rejectors, IL-4 fell significantly in the early post-transplant period (p=0.001) before rising significantly at the 1 week post-transplant time point, p=0.029 (although this level was still significantly below the pre-transplant baseline, p=0.006), and continuing to rise further at the 2 weeks post-transplant time point, p=0.003 (the level of IL-4 returning back to the pre-transplant baseline level at this time point, p=NS). This level of IL-4 gene expression was maintained at 4 weeks post-transplant (p=NS when comparing with the previous time point and with pre-transplant baseline).

In the rejectors, there was a similar significant fall in IL-4 gene expression at the early post-transplant time point (p=0.002). However, at the time of acute rejection (pre-anti-rejection therapy time point), there was a significant rise in IL-4 gene expression (p=0.023) above the early post-transplant time point level, although this level was not significantly different from the pre-transplant baseline.

The potent effect of anti-rejection therapy on peripheral IL-4 gene expression can be seen by its dramatic fall at the early post-anti-rejection therapy time point to a level not only significantly below the pre-anti-rejection therapy level (p=0.022), but also significantly below the pre-transplant baseline (p=0.015). This suppression of IL-4 following anti-rejection therapy was then followed by a significant rise back to the pre-transplant baseline 1 week after anti-rejection therapy (p=0.019), which remained unchanged by the 4 weeks post-anti-rejection therapy time point (p=NS for both time points when compared with the pre-transplant baseline). In addition, the levels of IL-4 gene expression at both these time points were not significantly different from the pre-anti-rejection therapy baseline.

Figure 6.1.3a

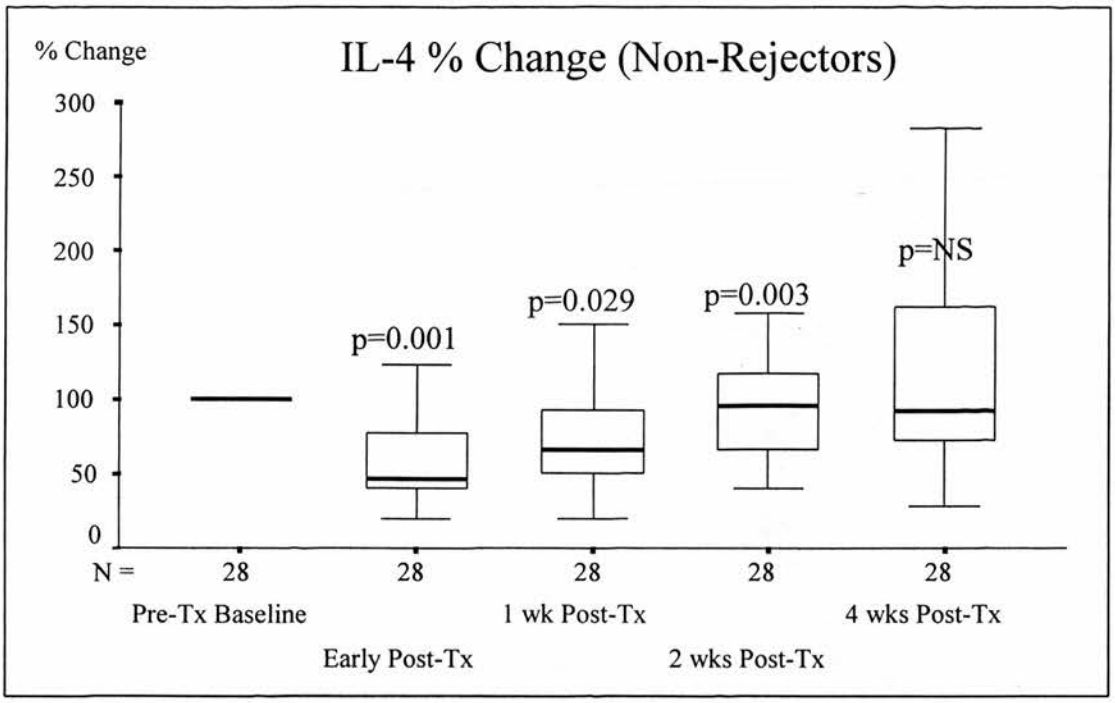


Figure 6.1.3b

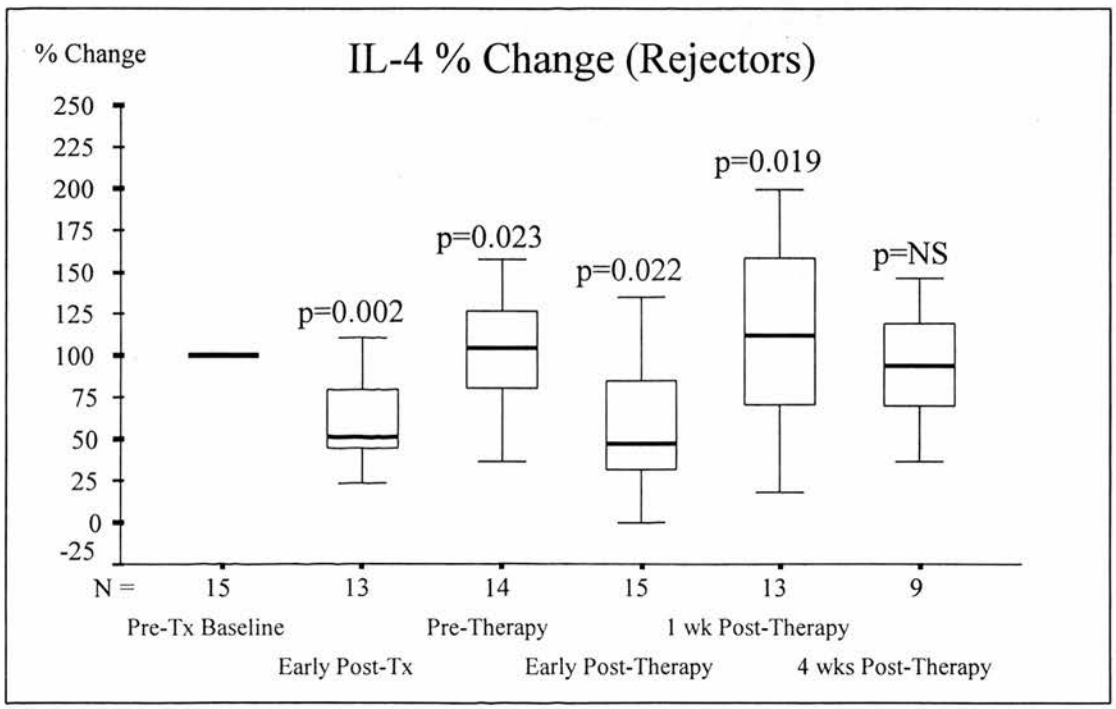


Figure 6.1.3 - Percentage change in IL-4 gene expression in non-rejectors and rejectors

6.1.4 IL-10 (figure 6.1.4)

The changes in peripheral T cell IL-10 gene expression appeared to be in the opposite direction to the changes seen in IL-4. In both non-rejectors and rejectors, there was a significant increase in IL-10 gene expression at the early post-transplant time point ($p=0.002$ and 0.004 respectively). In the non-rejectors, IL-10 remained elevated above the pre-transplant baseline at all subsequent post-transplant time points ($p=0.008$, 0.041 and 0.001 respectively for 1 week, 2 weeks & 4 weeks post-transplant time points compared with pre-transplant baseline). Interestingly, there was a further significant increase in IL-10 gene expression at the 4 weeks post-transplant time point from the 2 weeks post-transplant level in the non-rejectors ($p=0.045$).

In the rejectors, although there was no significant difference in the level of IL-10 gene expression between the pre-anti-rejection therapy and the early post-transplant time points (which was significantly higher than the pre-transplant baseline as mentioned in the previous paragraph), it is interesting that the level of IL-10 at the time of rejection was nevertheless not significantly different from the pre-transplant baseline. Although there were no further significant sequential changes in the level of IL-10 gene expression following anti-rejection therapy, the early post-anti-rejection therapy level was nevertheless significantly higher than the pre-transplant baseline ($p=0.005$). However, peripheral IL-10 gene expression at 1 week and 4 weeks post-anti-rejection therapy time points were not significantly different from the pre-transplant baseline.

Finally, there were no significant differences between the level of IL-10 at all the post-anti-rejection therapy time points when compared with the pre-anti-rejection therapy time point.

Figure 6.1.4a

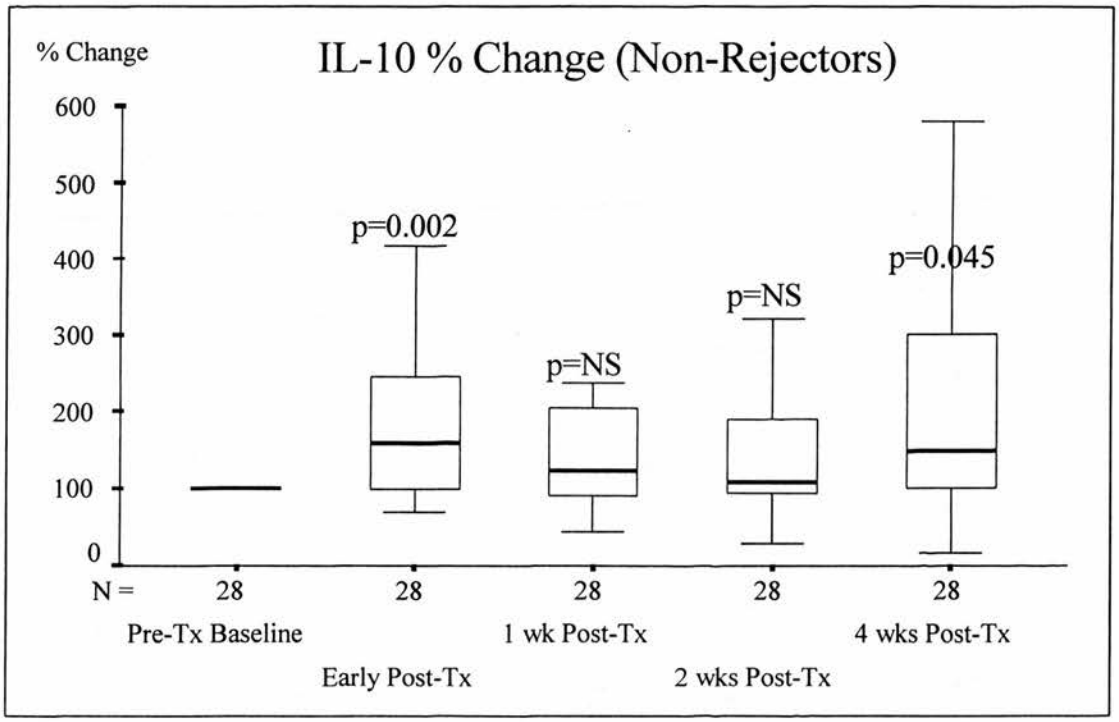


Figure 6.1.4b

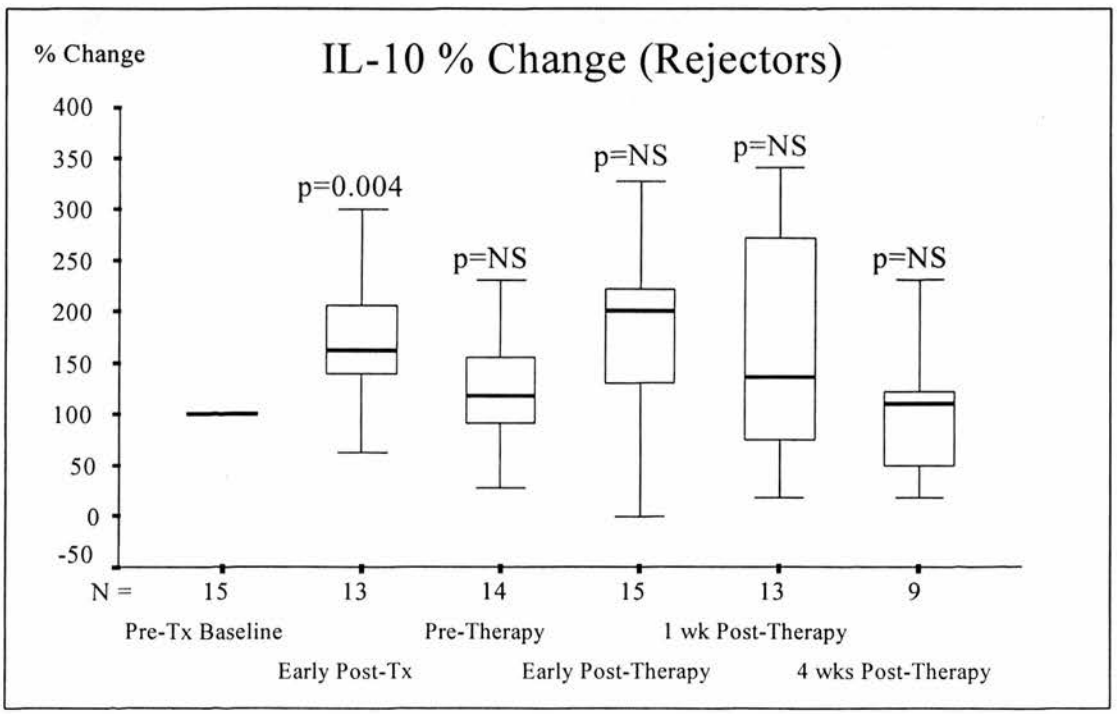


Figure 6.1.4 - Percentage change in IL-10 gene expression in non-rejectors and rejectors

6.1.5 IL-5 (figure 6.1.5)

In the sequential analysis, there was a significant fall in IL-5 gene expression from the pre-transplant baseline at the early post-transplant time point in both non-rejectors ($p=0.012$) and rejectors ($p=0.028$), but there were no further significant sequential changes in IL-5 gene expression at all subsequent post-transplant time points in both groups of patients.

In the non-rejectors, IL-5 remained significantly suppressed below the pre-transplant baseline at all the other post-transplant time points ($p=0.001$, 0.001 and 0.009 for 1 week, 2 weeks and 4 weeks post-transplant respectively). In the rejectors, although no significant increase in IL-5 expression was detectable at the pre-anti-rejection therapy time point compared with the early post-transplant time point (which was suppressed from the pre-transplant baseline), IL-5 expression at the pre-anti-rejection therapy time point was nevertheless not significantly different from the pre-transplant baseline.

However, following the completion of anti-rejection therapy, IL-5 gene expression had dropped significantly below the pre-transplant baseline at the early post-anti-rejection therapy time point ($p=0.008$), and remained significantly below the pre-transplant baseline at all the subsequent post-anti-rejection therapy time points ($p=0.046$ and 0.038 respectively at 1 and 4 weeks post-anti-rejection therapy time points), which was the same pattern as the IL-5 gene expression profile for non-rejectors described in the previous paragraph.

Although the fall in IL-5 gene expression at the early post-anti-rejection therapy time point from the pre-anti-rejection therapy time point was just outside statistical significance ($p=0.056$), the levels of IL-5 at 1 and 4 weeks post-anti-rejection therapy time points were nevertheless significantly below the level at the pre-anti-rejection therapy time point ($p=0.019$ and 0.038 respectively).

Figure 6.1.5a

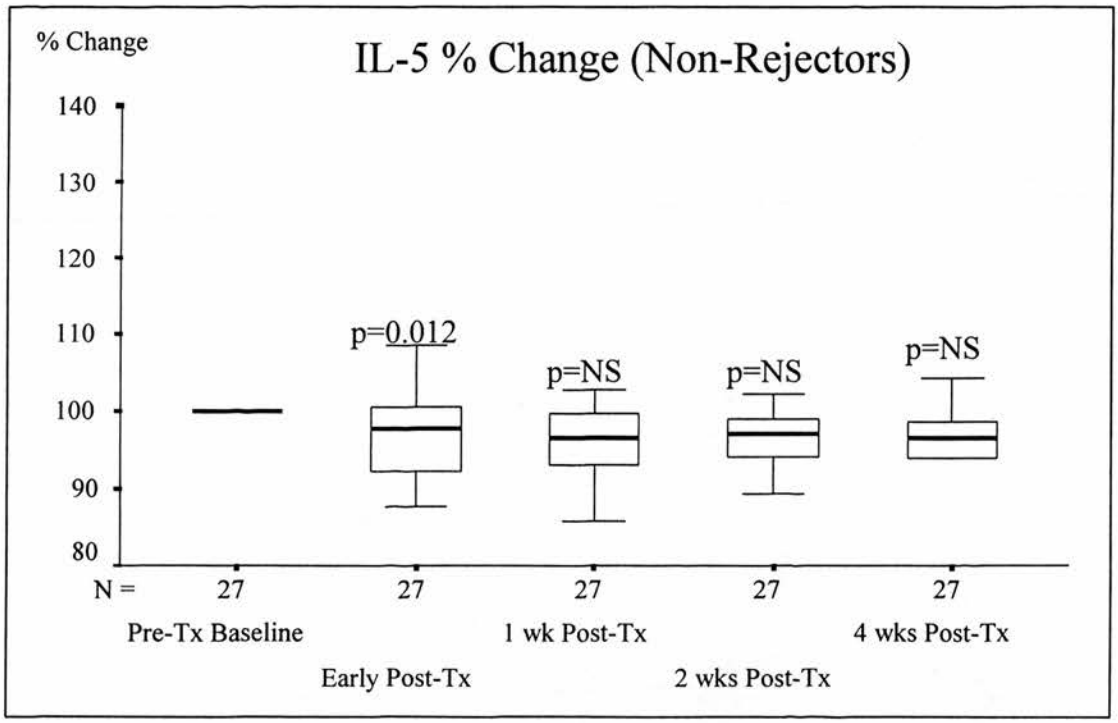


Figure 6.1.5b

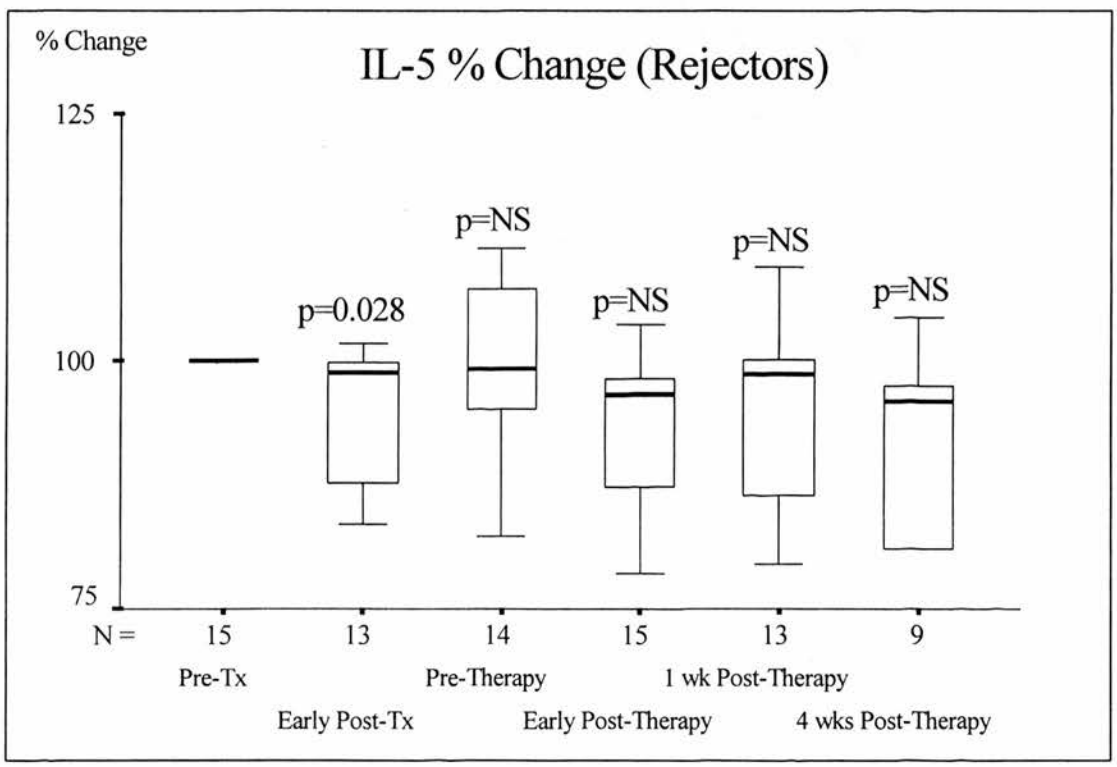


Figure 6.1.5 - Percentage change in IL-5 gene expression in non-rejectors and rejectors

6.1.6 IL-13 (figure 6.1.6)

In the non-rejectors, there were no significant changes in the level of IL-13 gene expression either sequentially from one time point to another or when all post-transplant time points were compared with the pre-transplant baseline.

However, in the rejectors, although IL-13 expression at the early post-transplant time point was not significantly different from the pre-transplant baseline (as in the non-rejectors), there was a significant rise in the level of IL-13 at the time of rejection (the pre-anti-rejection therapy time point) both above the pre-transplant baseline ($p=0.013$) and the early post-transplant time point ($p=0.003$). At the early post-anti-rejection therapy time point, IL-13 gene expression had fallen significantly below the pre-anti-rejection therapy level ($p=0.048$) to the pre-transplant baseline (p=NS), and it remained at the pre-transplant level at both 1 and 4 weeks post-anti-rejection therapy time points (p=NS for both time points when compared sequentially, with the pre-transplant baseline, or with the pre-anti-rejection therapy time point).

6.1.7 GrB (figure 6.1.7)

In the non-rejectors, there were no significant differences in the level of GrB gene expression at all sequential time points from pre-transplant to 2 weeks post-transplant. However, the level of GrB at 1 and 2 weeks post-transplant time points were both significantly lower than the pre-transplant baseline ($p=0.003$ and 0.011 respectively). Interestingly, at 4 weeks post-transplant, GrB level rose significantly from 2 week post-transplant ($p=0.039$) back to the pre-transplant level (p=NS).

In the rejectors, unlike in the non-rejectors, there was a significant fall in GrB gene expression at the early post-transplant time point ($p=0.002$), but there were no significant differences at all subsequent time points when compared with each other sequentially, with the pre-transplant baseline, or with the pre-anti-rejection therapy time point.

Figure 6.1.6a

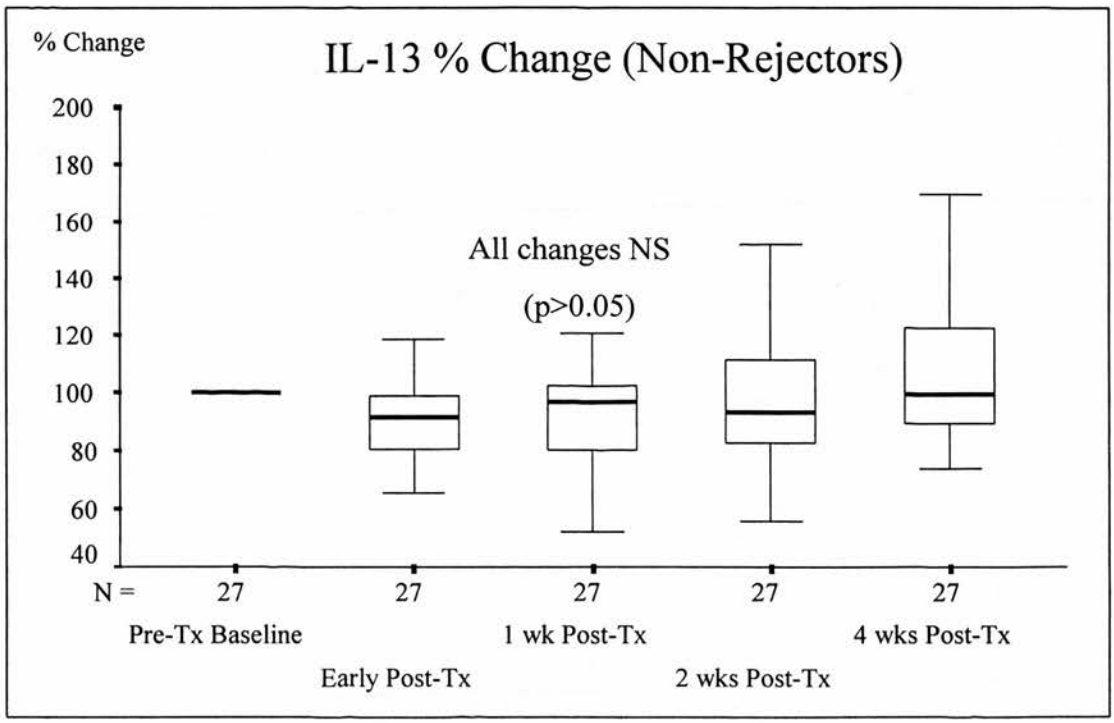


Figure 6.1.6b

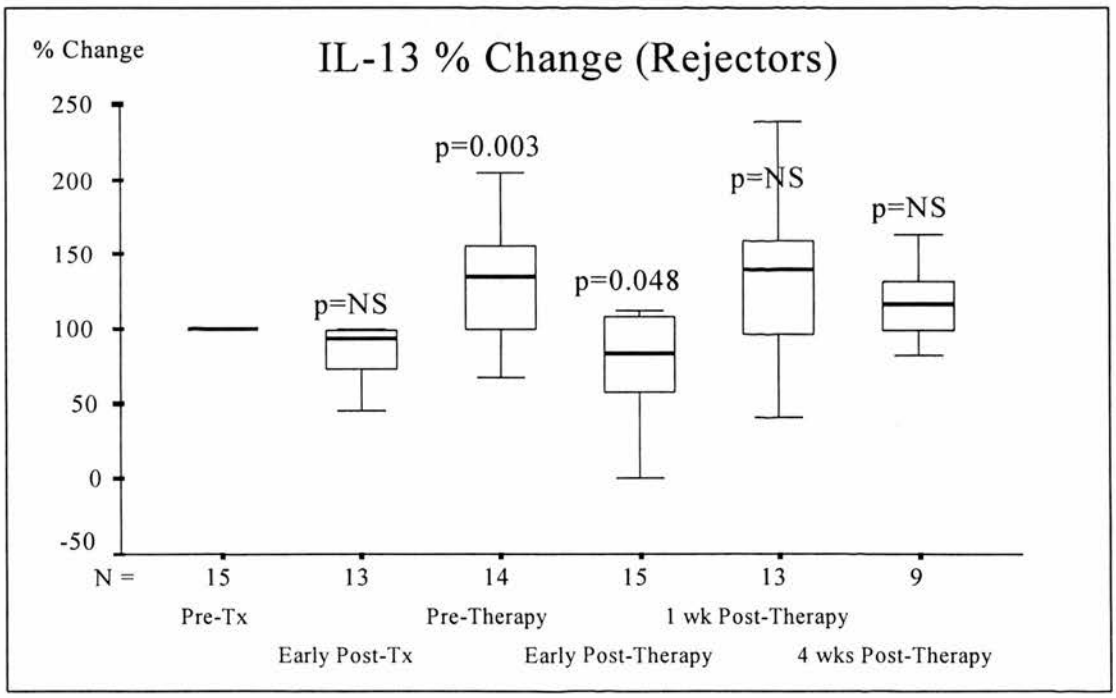


Figure 6.1.6 - Percentage change in IL-13 gene expression in non-rejectors and rejectors

Figure 6.1.7a

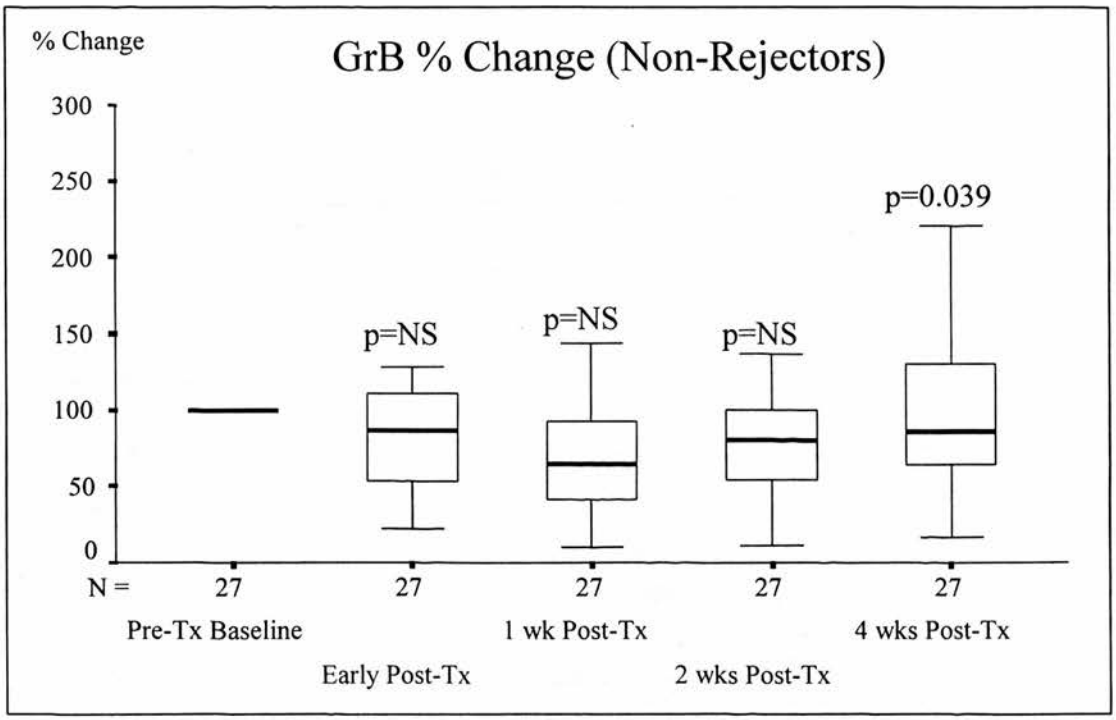


Figure 6.1.7b

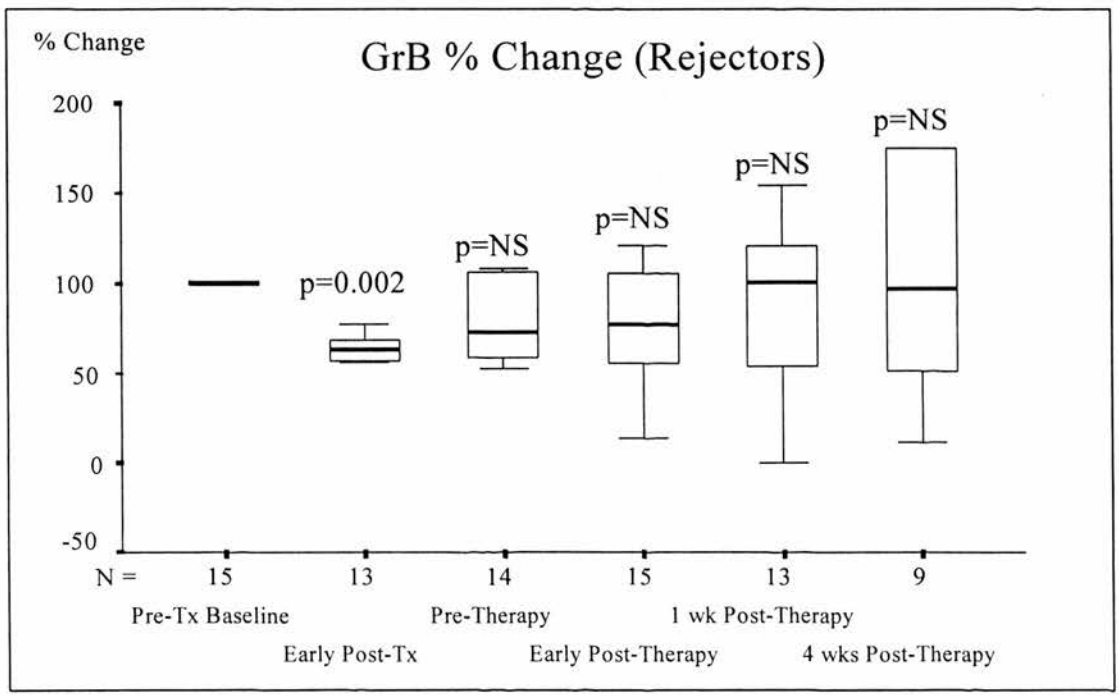


Figure 6.1.7 - Percentage change in GrB gene expression in non-rejectors and rejectors

6.1.8 FasL (figure 6.1.8)

The sequential pattern of changes in FasL gene expression in non-rejectors has similarities with the pattern of changes seen for GrB. There were no significant changes sequentially from pre-transplant to 2 weeks post-transplant. At the 4 weeks post-transplant time point, a significant rise in FasL gene expression above the level at 2 weeks post-transplant was evident ($p=0.008$), but this level was not significantly different from the pre-transplant baseline. However, unlike GrB, only the FasL level at 1 week post-transplant was significantly below the pre-transplant baseline ($p=0.032$).

In the rejectors, there were no significant differences in the level of FasL between all the post-transplant time points when compared with each other sequentially, with the pre-transplant baseline, or with the pre-anti-rejection therapy time point.

6.2 Analysis of cytokine gene expression ratio profiles in patients with and without early acute rejections

From the previous section, it was demonstrated that IL-4 and IL-10 gene expression profiles appeared to be opposing in pattern. Hence, the ratio of IL-10 to IL-4 was examined to assess the balance between these two Th2 cytokines by taking the ratios of the raw RT-PCR ELISA data of these two Th2 cytokines, and this is shown in figure 6.2.1. As in the previous section, the graphs for non-rejectors, (a), and rejectors, (b), are set next to each other within each figure in this section. We also analysed the balance between Th1 and Th2 cytokines by taking similar ratios between Th2 (IL-4 and IL-10) to Th1 cytokines (IL-2 and IFN-γ). The profile of changes in these ratios are shown in figures 6.2.2 to 6.2.5. Finally, for completeness, the ratio of IFN-γ to IL-2 was also taken, and this is shown in figure 6.2.6. It was appropriate for these ratios to be taken as all the 4 cytokines were analysed on the same RT-PCR ELISA run for each patient.

Figure 6.1.8a

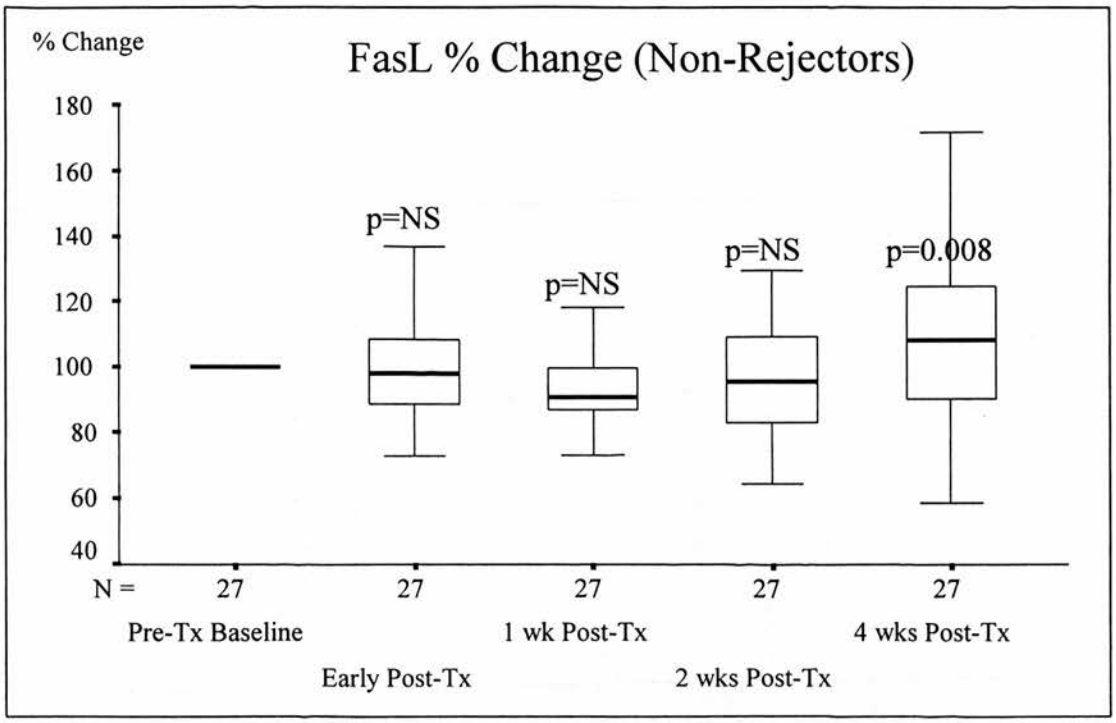


Figure 6.1.8b

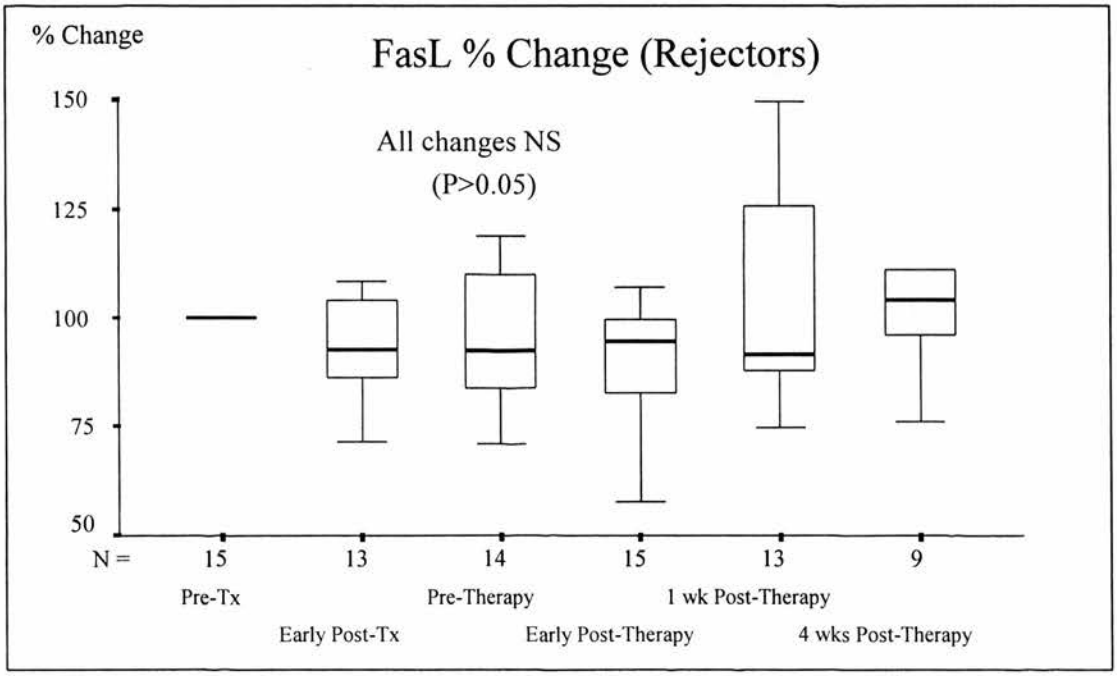


Figure 6.1.8 - Percentage change in FasL gene expression in non-rejectors and rejectors

6.2.1 IL-10/IL-4 Ratio (figure 6.2.1)

The sequential changes in IL-10/IL-4 ratios in both non-rejectors and rejectors were the most prominent of all the cytokine ratios taken. In the non-rejectors, there was a highly significant rise in the ratio at the early post-transplant time point ($p<0.001$). Although IL-10/IL-4 ratio fell significantly and sequentially at 1 and 2 weeks post-transplant ($p=0.003$ and 0.043 respectively) before settling down at 4 weeks post-transplant (p=NS between 2 and 4 weeks post-transplant ratios), all the IL-10/IL-4 ratios at 1, 2 and 4 weeks post-transplant time points remained significantly higher than the pre-transplant baseline ($p<0.001$, $p=0.038$ and 0.003 respectively).

In the rejectors, there was a similar significant rise in IL-10/IL-4 ratio at the early post-transplant time point ($p=0.002$). This ratio fell significantly at the time of rejection (pre-anti-rejection therapy time point, $p=0.003$) to a level not significantly different from the pre-transplant baseline, a distinct deviation from the consistently elevated IL-10/IL-4 ratios seen in the non-rejectors' profile.

At the early post-anti-rejection therapy time point, IL-10/IL-4 ratio rose significantly above both the pre-anti-rejection therapy ($p=0.003$) and pre-transplant ($p=0.001$) levels, before falling back significantly at the 1 week post-anti-rejection therapy ($p=0.006$) and plateauing out at the 4 weeks post-anti-rejection therapy time point to the pre-transplant baseline level (p=NS when the ratios at 1 and 4 weeks post-anti-rejection time points were compared with each other and with the pre-transplant baseline).

As IL-10/IL-4 ratio at the pre-anti-rejection therapy time point was not significantly different from the pre-transplant baseline, it was not surprising therefore that both the ratios at 1 and 4 weeks post-anti-rejection therapy time points were not significantly different from the pre-anti-rejection therapy time point either.

Figure 6.2.1a

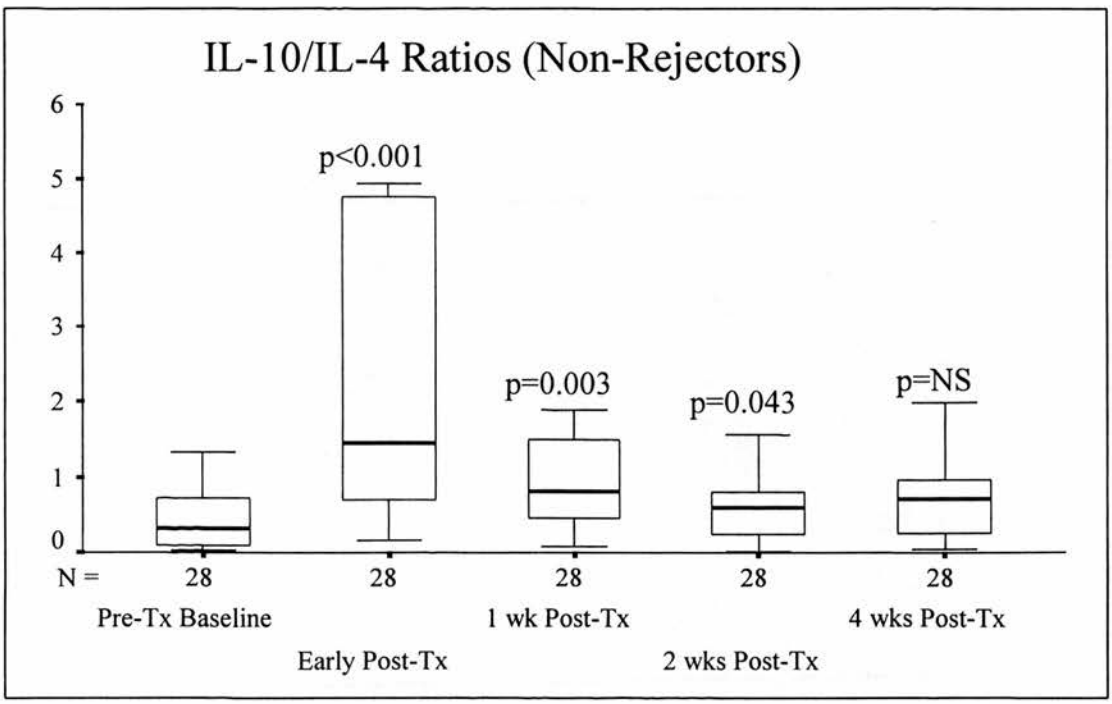


Figure 6.2.1b

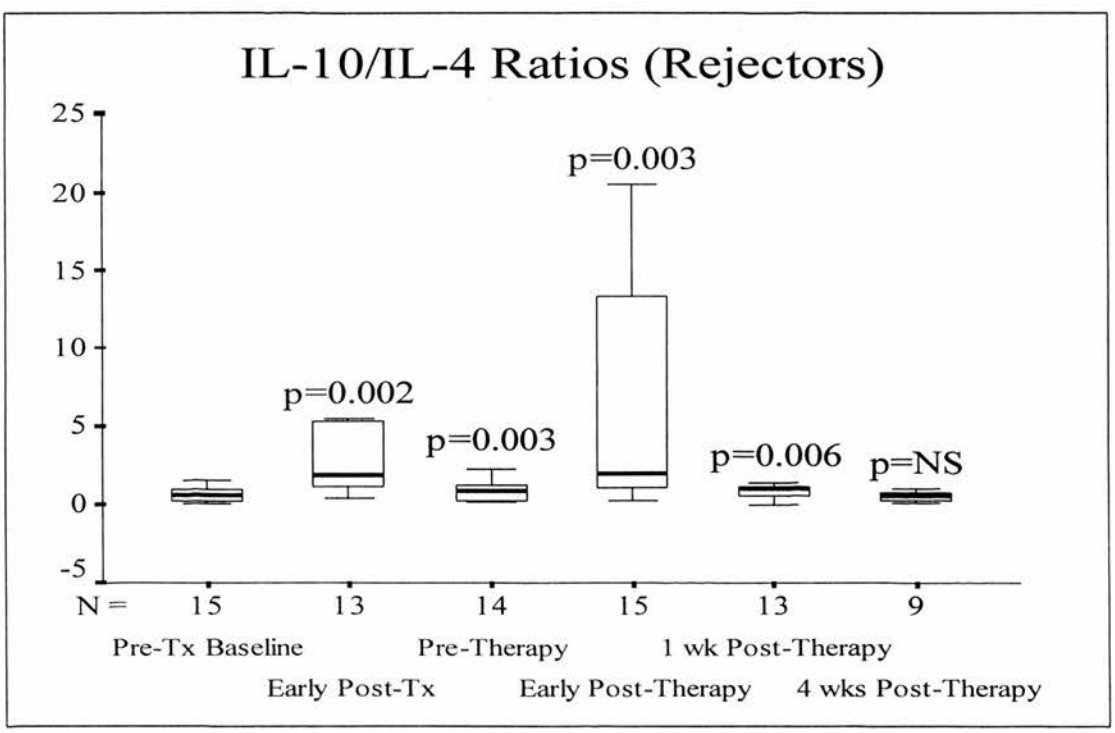


Figure 6.2.1 - IL-10/IL-4 ratios in non-rejectors and rejectors

6.2.2 IL-4/IL-2 Ratio (figure 6.2.2)

In the non-rejectors, the IL-4/IL-2 ratio fell significantly below the pre-transplant baseline in the early post-transplant time point ($p=0.011$) and this ratio rose significantly at 1 week post-transplant ($p=0.036$) back to the pre-transplant baseline ($p=NS$). There were no further significant changes in the remaining post-transplant time points when compared sequentially with each other or with the pre-transplant baseline.

However, in the rejectors, no significant change in IL-4/IL-2 ratio was found at the early post-transplant time point. At the time of rejection (pre-anti-rejection therapy time point), this ratio rose significantly both above the early post-transplant time point ($p=0.034$) and the pre-transplant baseline ($p=0.016$). There were no further sequential changes in the IL-4/IL-2 ratio in all the subsequent post-anti-rejection therapy time points. Although the ratio at the early post-anti-rejection therapy time point was not significantly different from the pre-transplant baseline, the ratio at 1 and 4 weeks post-anti-rejection therapy time points were significantly higher than the pre-transplant baseline ($p=0.011$ and 0.028 respectively). There were no significant differences between the IL-4/IL-2 ratios of any of the post-anti-rejection therapy time points and the pre-anti-rejection therapy time point.

6.2.3 IL-4/IFN-γ Ratio (figure 6.2.3)

The pattern of changes in IL-4/IFN-γ ratios was similar to that seen for IL-4 in the non-rejectors, with a highly significant initial fall in the ratio at the early post-transplant time point ($p<0.001$) and then a steady rise in the ratio at 1 week ($p=0.026$) and 2 weeks ($p=0.05$) post-transplant back to the pre-transplant level (the IL-4/IFN-γ ratio was not significantly different to the pre-transplant ratio at both time points). The IL-4/IFN-γ ratio at the 4 weeks post-transplant time point was maintained at the pre-transplant baseline level ($p=NS$ compared with previous time point and with pre-transplant baseline).

Figure 6.2.2a

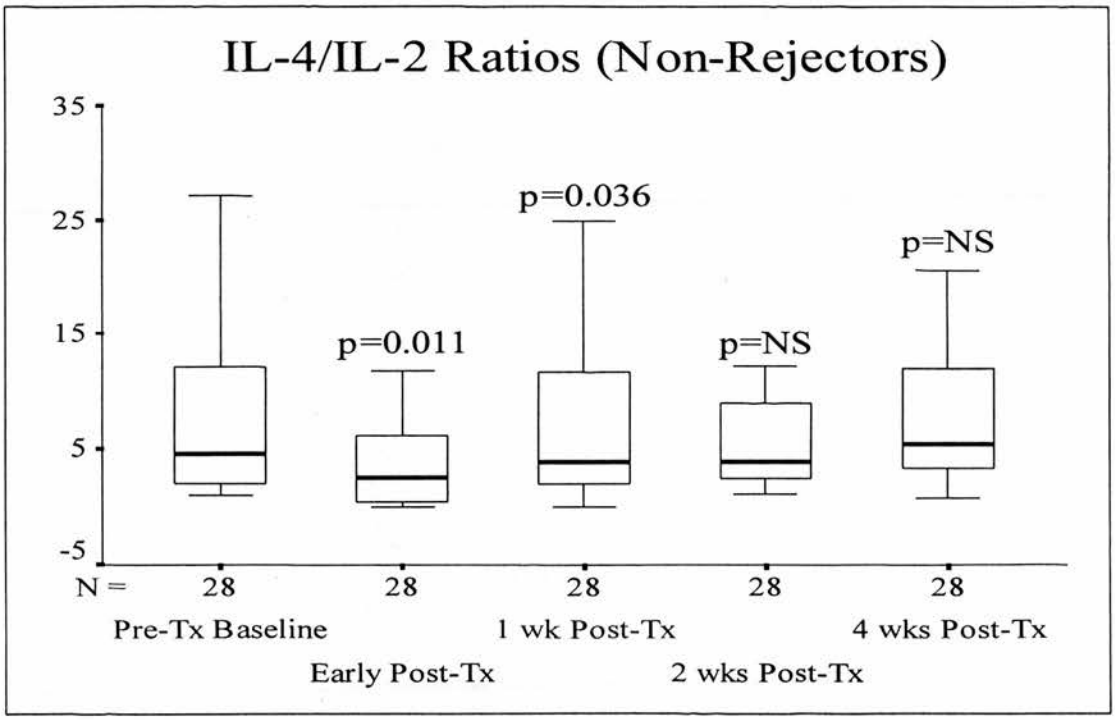


Figure 6.2.2b

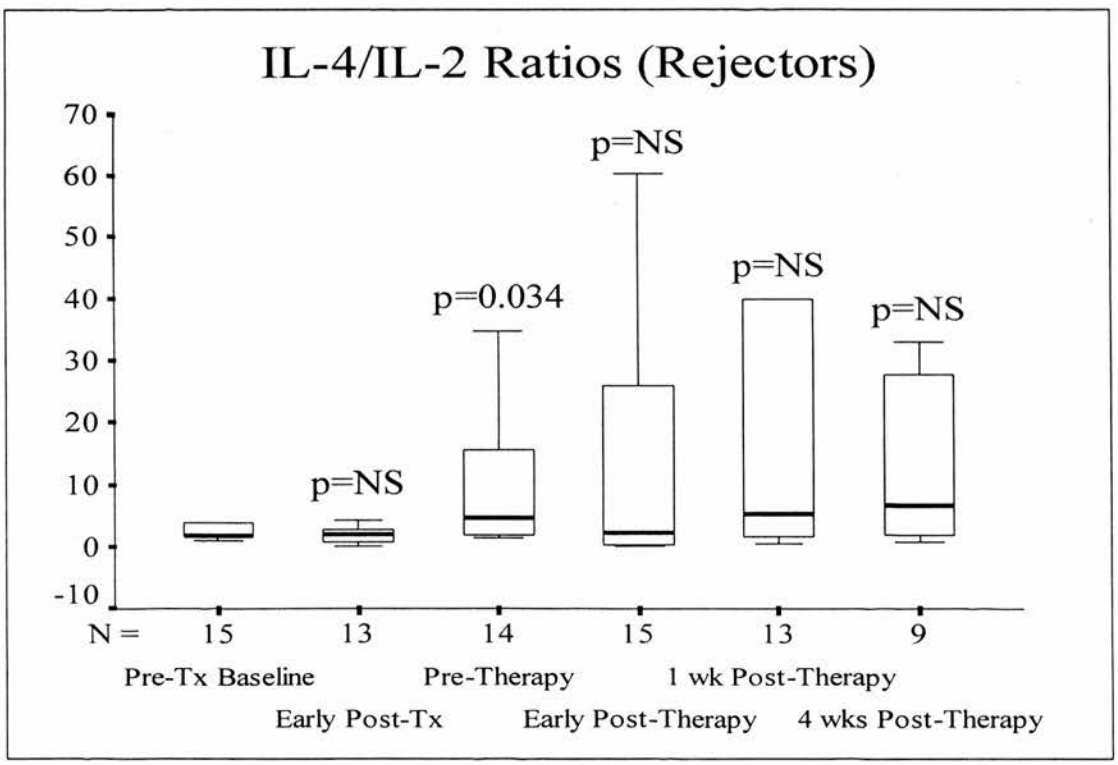


Figure 6.2.2 - IL-4/IL-2 ratios in non-rejectors and rejectors

Figure 6.2.3a

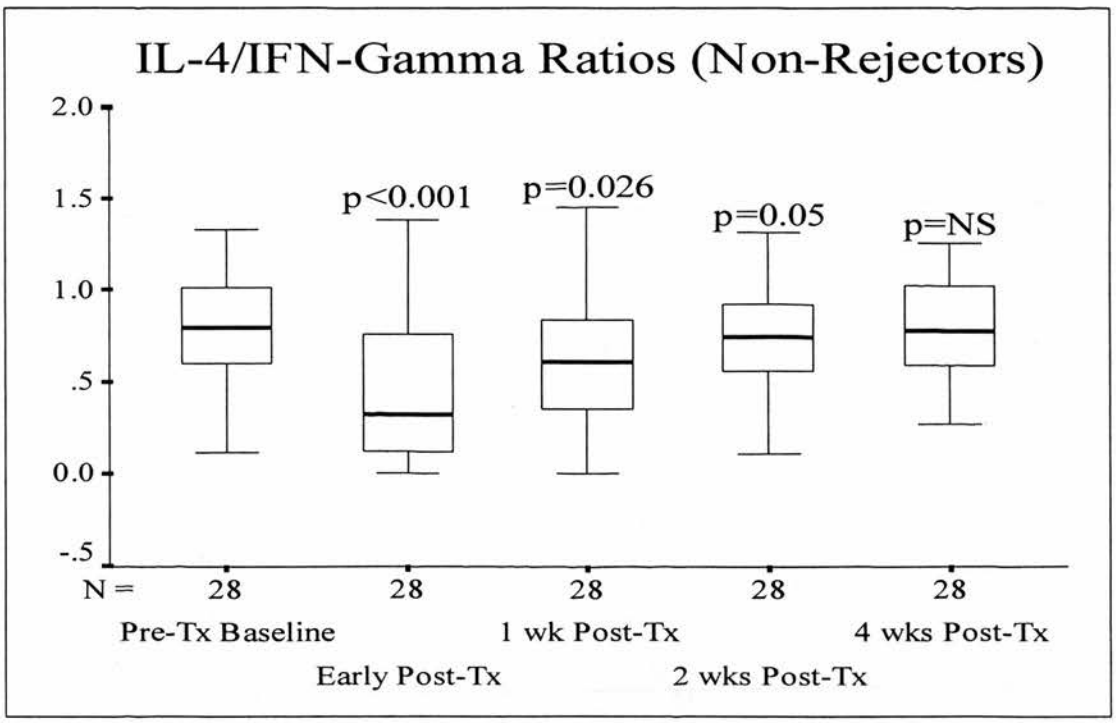


Figure 6.2.3b

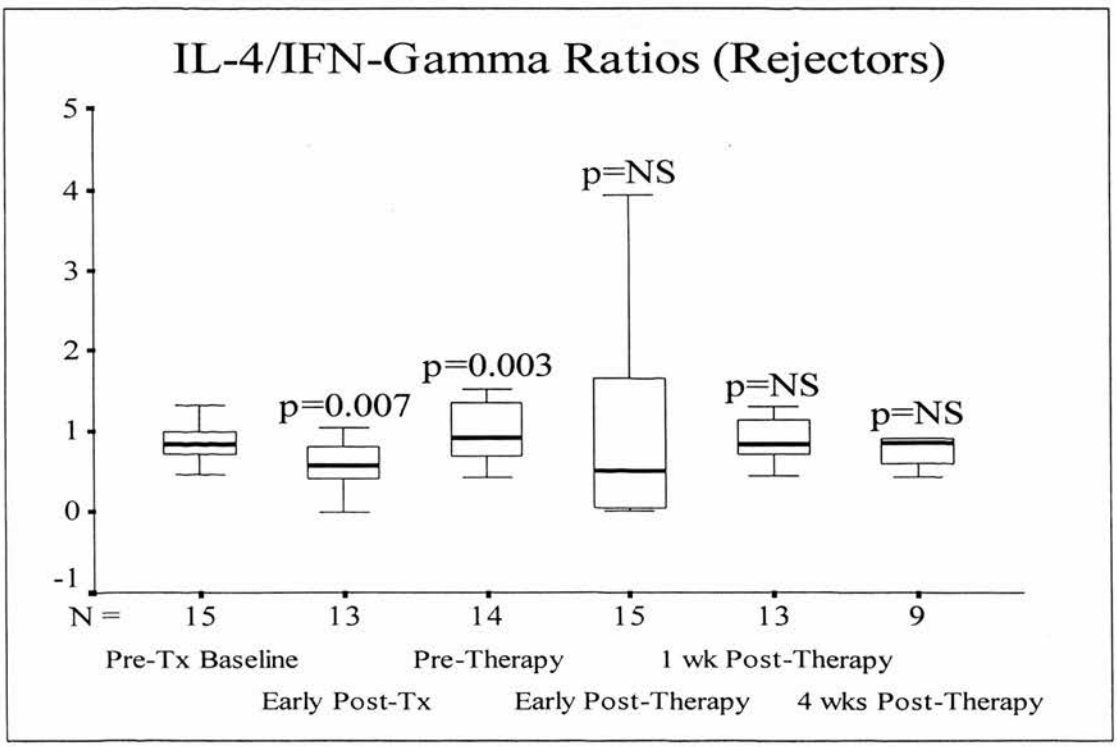


Figure 6.2.3 - IL-4/IFN-γ ratios in non-rejectors and rejectors

In the rejectors, there was a similar significant fall in IL-4/IFN-γ ratio at the early post-transplant time point (p=0.007). At the pre-anti-rejection therapy time point, there was a significant rise in the ratio from the early post-transplant time point (p=0.003) back to the pre-transplant baseline ratio (p=NS when the pre-anti-rejection therapy time point was compared with the pre-transplant baseline). There were no significant differences in IL-4/IFN-γ ratios at all the post-anti-rejection therapy time points when compared sequentially with each other or with either the pre-transplant or pre-anti-rejection therapy baselines.

6.2.4 IL-10/IL-2 Ratio (figure 6.2.4)

In the non-rejectors, a significant rise in IL-10/IL-2 ratio was seen at the early post-transplant time point (p=0.001) but there were no further significant sequential changes in the ratios at all subsequent post-transplant time points. The IL-10/IL-2 ratio remained significantly above the pre-transplant baseline ratio at 1 week (p<0.001) and 4 weeks (p=0.006) post-transplant time points but was not significantly different from the pre-transplant baseline at 2 weeks post-transplant.

IL-10/IL-2 ratios rose significantly at the early post-transplant time point (p=0.001) in the rejectors, but there was no significant difference in the ratio at the time of rejection (pre-anti-rejection therapy time point) compared with the early post-transplant time point, although this ratio was still significantly higher than the pre-transplant baseline (p=0.002). There were no significant changes in IL-10/IL-2 ratios in all the post-anti-rejection therapy time points when compared sequentially with each other or with the pre-anti-rejection therapy time point as the second baseline. However, the IL-10/IL-2 ratios at the early and 1 week post-anti-rejection therapy time points were significantly higher than the pre-transplant baseline (p=0.001 and 0.019 respectively), but there was no significant difference in IL-10/IL-2 ratio at the 4 weeks post-anti-rejection time point when compared with the pre-transplant baseline ratio.

Figure 6.2.4a

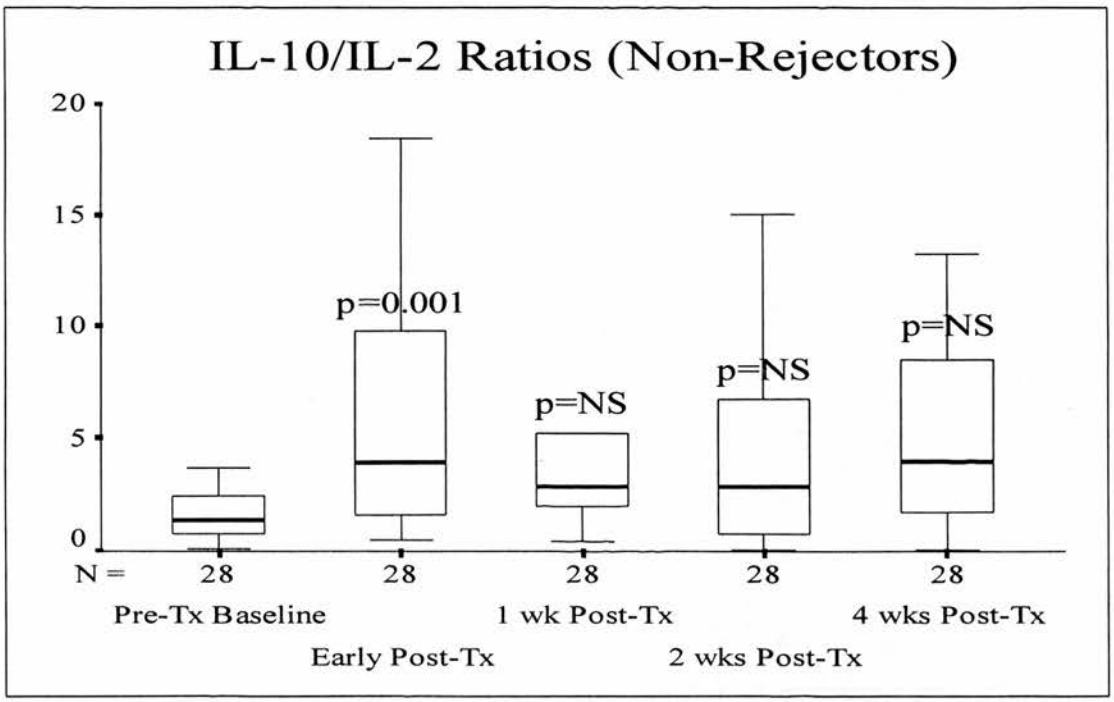


Figure 6.2.4b

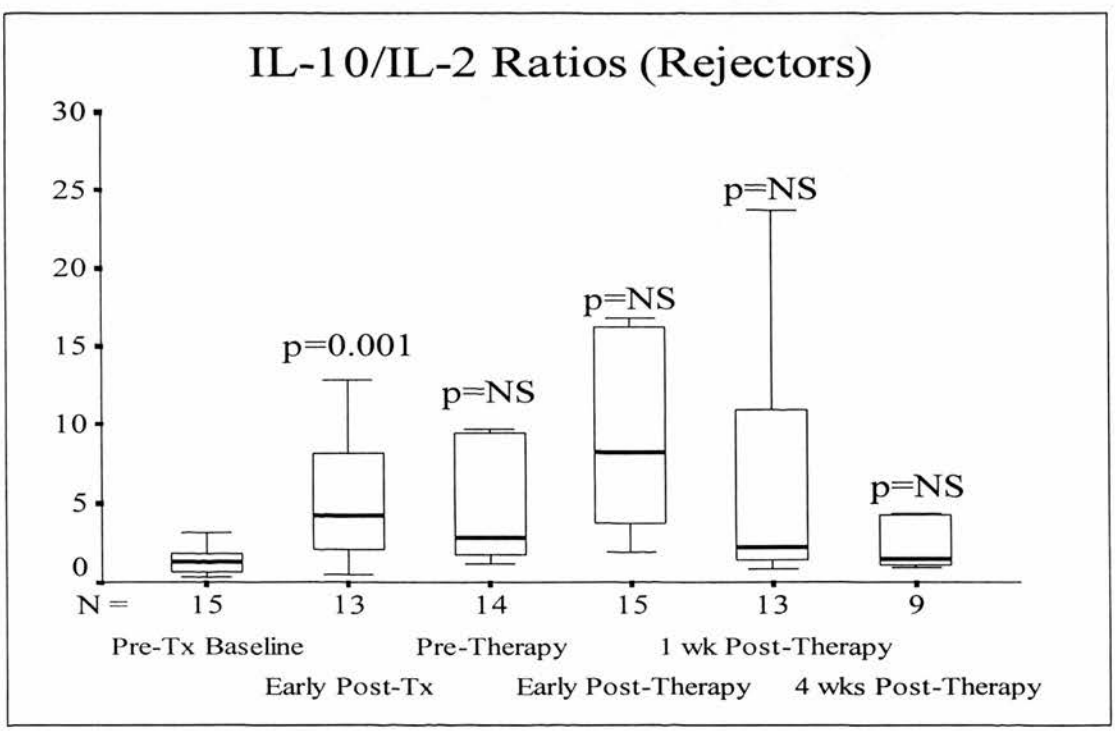


Figure 6.2.4 - IL-10/IL-2 ratios in non-rejectors and rejectors

6.2.5 IL-10/IFN-γ Ratio (figure 6.2.5)

The overall non-rejectors' IL-10/IFN-γ ratio profile was similar to their IL-10/IL-2 ratio profile, with a significant rise in IL-10/IFN-γ ratio at the early post-transplant time point (p=0.001) and no further significant sequential changes in the ratio at all subsequent post-transplant time points. IL-10/IFN-γ ratios, like IL-10/IL-2 ratios, remained significantly above the pre-transplant baseline ratio at 1 week and 4 weeks post-transplant (p=0.006 for both time points) but was not significantly different from the pre-transplant baseline at the 2 weeks post-transplant time point.

The rejectors' IL-10/IFN-γ ratio profile on the other hand was quite different to the profile for IL-10/IL-2 ratio. The IL-10/IFN-γ ratio rose significantly at the early post-transplant time point (p=0.004) in the rejectors, but there was no significant difference in the ratio at the time of rejection (pre-anti-rejection therapy time point) compared with the early post-transplant time point, although this ratio was still significantly higher than the pre-transplant baseline (p=0.026).

Although there was no significant change in IL-10/IFN-γ ratio at the early post-anti-rejection therapy time point from the pre-anti-rejection therapy time point (this ratio was also significantly higher than the pre-transplant baseline, p=0.001), there was a significant sequential fall in the ratio at the 1 week (p=0.039) and 4 weeks (p=0.015) post-anti-rejection therapy time points back to the pre-transplant baseline ratio (p=NS when at both time points were compared with the pre-transplant baseline). However, there were no significant differences in the IL-10/IFN-γ ratio at all the post-anti-rejection time points when compared with the pre-anti-rejection therapy time point.

Figure 6.2.5a

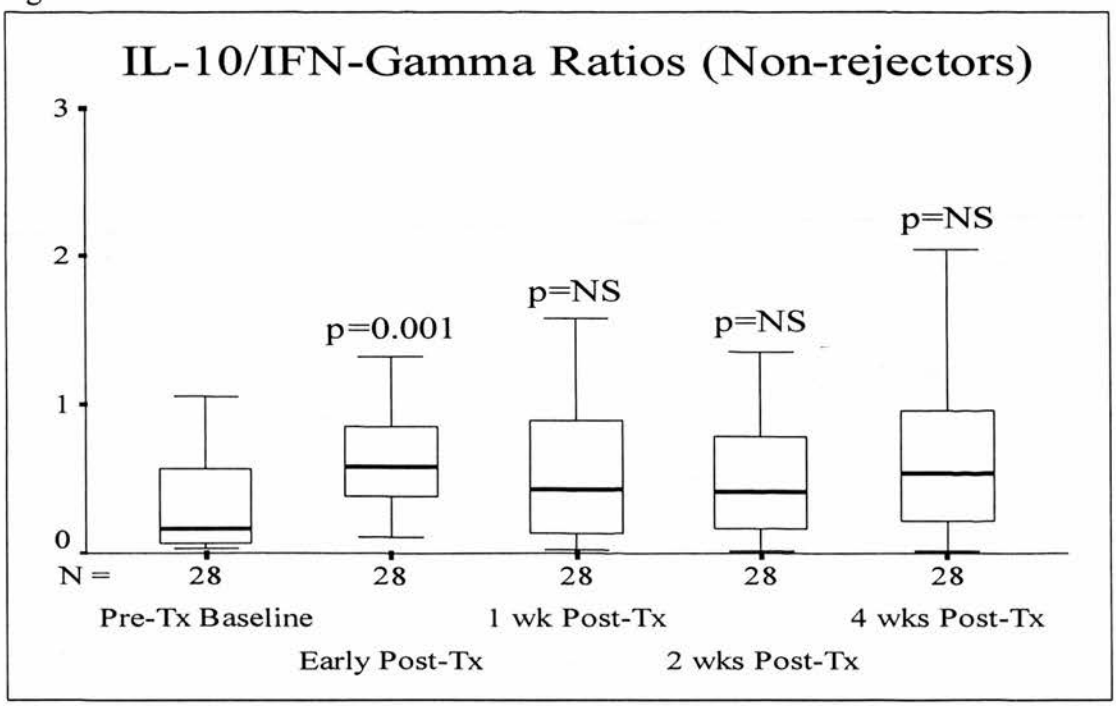


Figure 6.2.5b

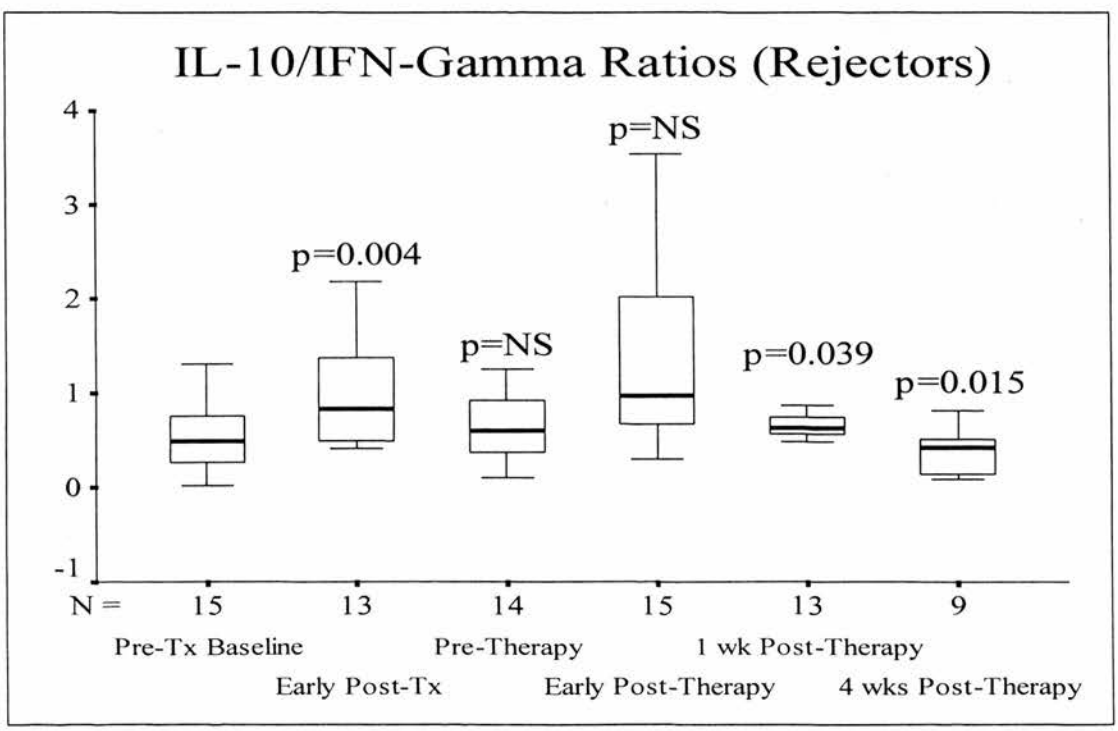


Figure 6.2.5 – IL-10/IFN-γ ratios in non-rejectors and rejectors

6.2.6 IFN-γ/IL-2 Ratio (figure 6.2.6)

In the non-rejectors, there were no significant changes in IFN-γ/IL-2 ratio when the time points were compared sequentially with each other or with the pre-transplant baseline.

In the rejectors, there were no significant changes in IFN-γ/IL-2 ratio when the time points were compared sequentially with each other either. Nevertheless, the ratios at the pre-anti-rejection therapy and all 3 post-anti-rejection therapy time points were significantly higher than the pre-transplant baseline ($p=0.026$, 0.009, 0.039 and 0.021 respectively), but there were no significant differences in the ratios when the post-anti-rejection therapy time points were compared with the pre-anti-rejection therapy time point.

6.3 Analysis of cytokine/CTL activation marker gene expression profiles and cytokine ratios at all protocol sampling time points prior to acute rejection

In this section, we looked at the data from all the protocol sampling time points prior to commencing anti-rejection therapy for patients who had experienced early acute rejection. We were interested to see if there were any significant differences in the individual cytokine/CTL activation marker gene expression profile and the profile of the cytokine ratios (as listed in the previous section) in the rejectors, at the time points prior to the occurrence of the acute rejection episode, when compared with the gene expression profile of the non-rejectors at the same time points.

As the number of protocol samples from 2 weeks post-transplant onwards in this group of patients were small, meaningful analysis could only be made on the data from the first 4 protocol sampling time points. Note that all pre-anti-rejection therapy samples which coincided with any of the protocol sampling time points had been

Figure 6.2.6a

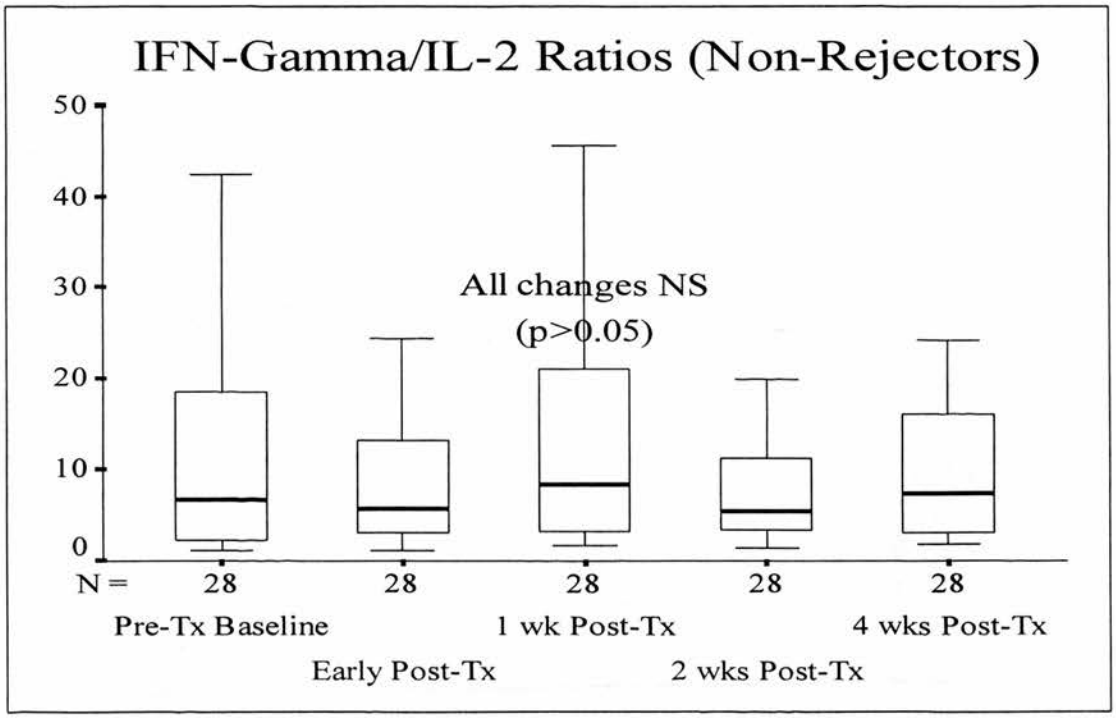


Figure 6.2.6b

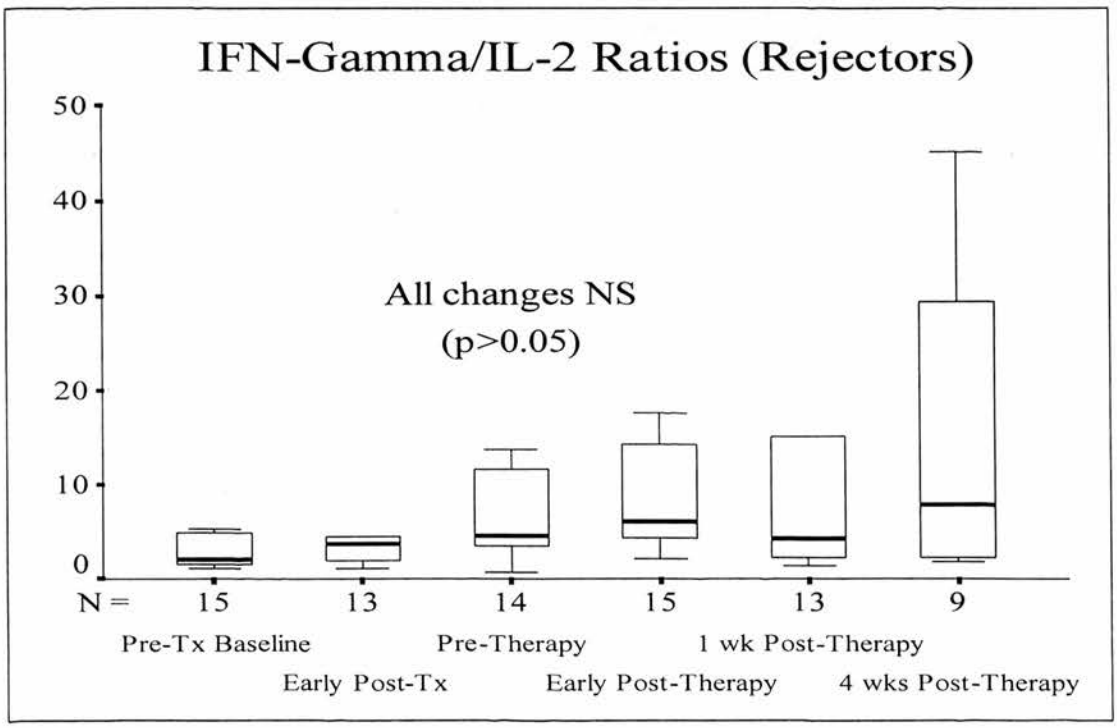


Figure 6.2.6 – IFN-γ/IL-2 ratios in non-rejectors and rejectors

included in this analysis (thus accounting for the difference in the number of early post-transplant samples shown in the rejector's graphs in the previous section compared with that shown in this section).

The scale of the y-axis in each graph for the rejectors had been set to the same scale as that for the non-rejectors in this section to facilitate the visual comparison between the two groups over the same protocol sampling time points, and to further improve the ease of comparison, the graphs for each cytokine/CTL activation marker in the non-rejectors over the first 4 protocol sampling time points (graph "a" of all the figures in sections 6.1 and 6.2) had been reproduced next to that for the rejectors. The results of the analyses are set out in figures 6.3.1 to 6.3.14 (graph "a" and "b" showing the profiles for rejectors and non-rejectors respectively). As in the previous section, the 2-tailed p values quoted in all the figures in this section were obtained using the Wilcoxon signed rank sum test for matched pairs comparing the data at one time point with the one immediately preceding it. The p values comparing each post-transplant time point with the pre-transplant baseline are quoted in the text. Only p values of significant differences will be quoted in the text and in the figures, otherwise the abbreviation "NS" will be used to denote all p values greater than 0.05.

6.3.1 IL-2 and IFN-γ (figures 6.3.1 and 6.3.2)

In the rejectors, the pre-rejection gene expression profiles for IL-2 and IFN-γ are similar. There was a significant fall in the level of IL-2 and IFN-γ gene expression at the early post-transplant time point ($p=0.011$ and 0.017 respectively), and this depressed level of both cytokines remained significantly below the pre-transplant baseline at 1 week post-transplant ($p=0.003$ and 0.001 respectively). Although there were no significant differences in the level of both cytokines between 1 week and 2 weeks post-transplant, both IL-2 and IFN-γ levels at 2 weeks post-transplant were nevertheless not significantly different from their respective pre-transplant baselines.

Figure 6.3.1a - Rejectors

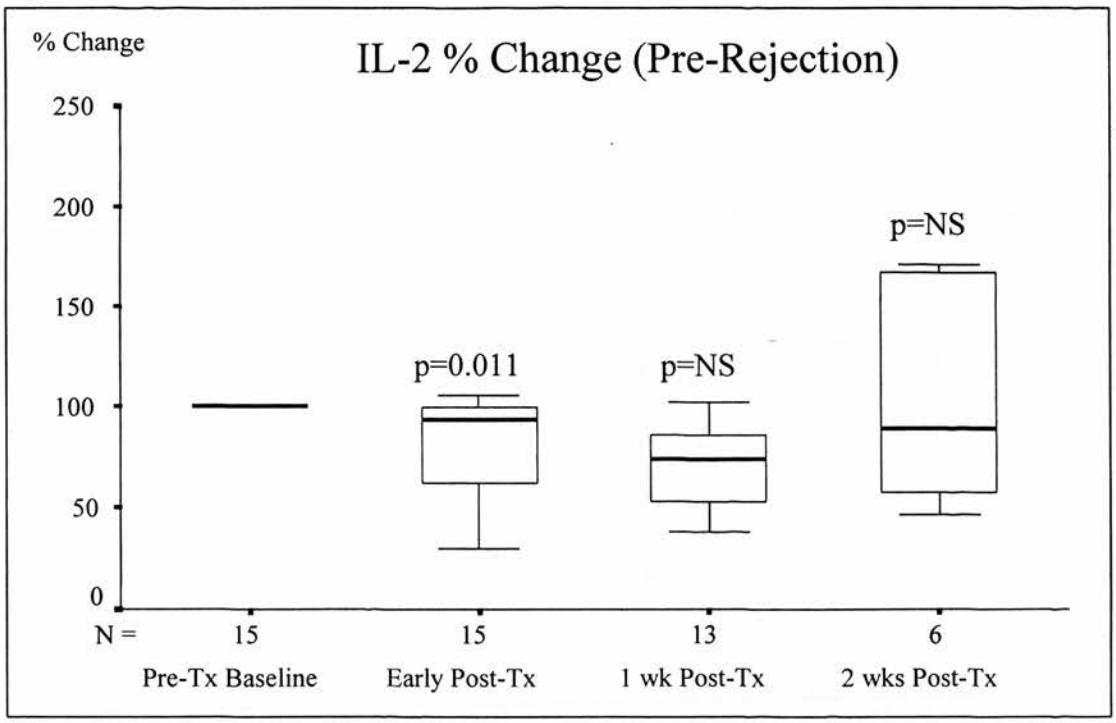


Figure 6.3.1b - Non-rejectors

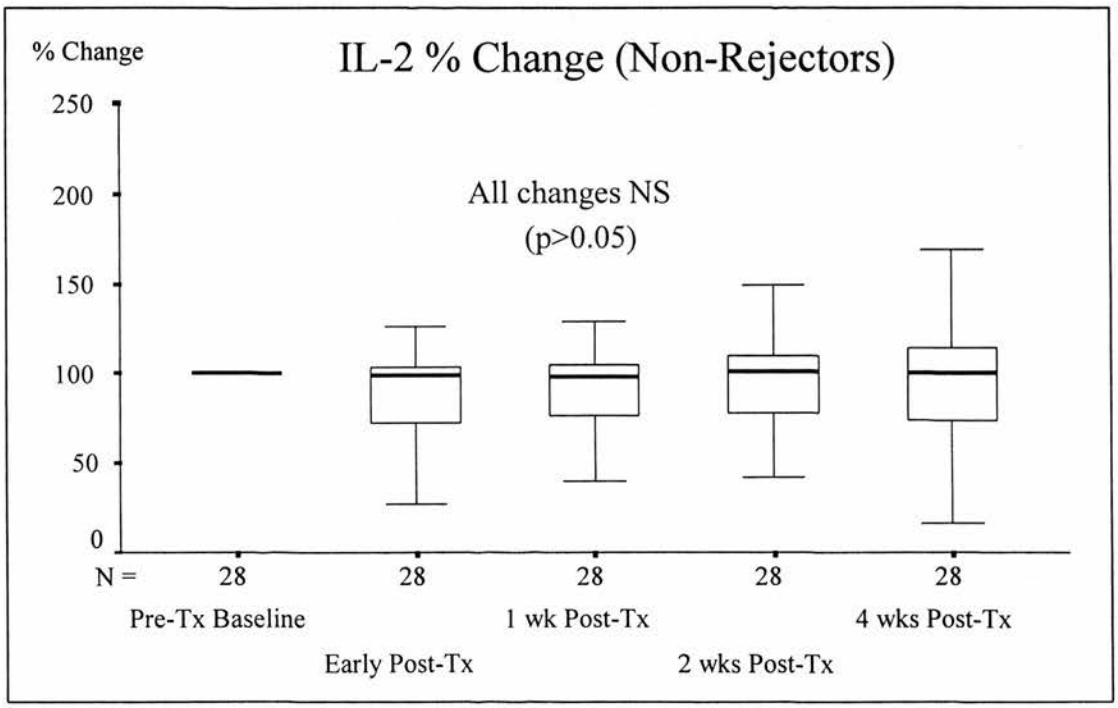


Figure 6.3.1 – Percentage change in IL-2 gene expression at pre-rejection time points in rejectors compared with non-rejectors over the same time points

Figure 6.3.2a - Rejectors

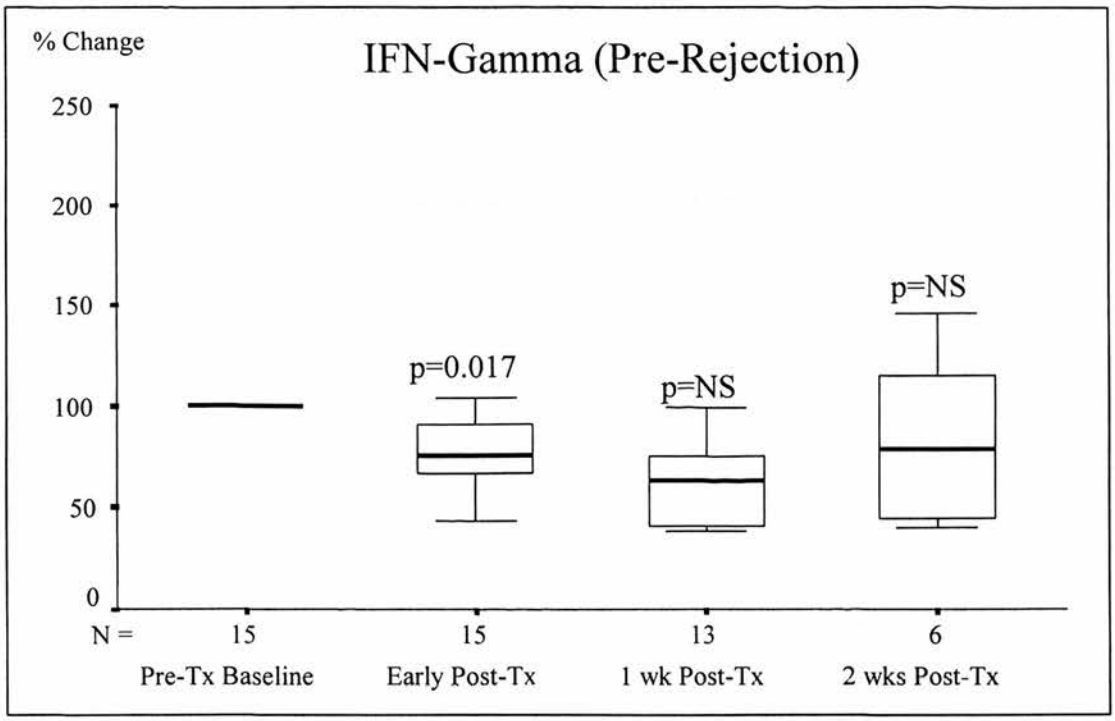


Figure 6.3.2b - Non-rejectors

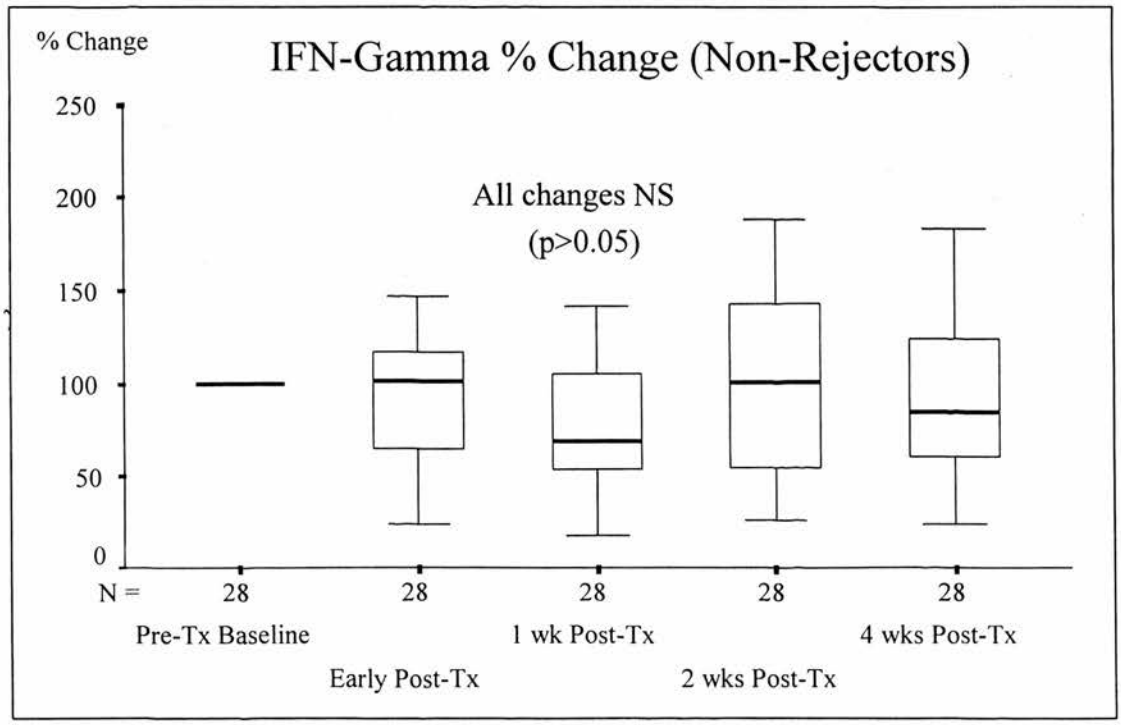


Figure 6.3.2 – Percentage change in IFN-γ gene expression at pre-rejection time points in rejectors compared with non-rejectors over the same time points

In contrast, both IL-2 and IFN-γ gene expression profiles in the non-rejectors (figures 6.3.1b and 6.3.2b) were rather "flat" with no significant differences both sequentially from one time point to the next and when all post-transplant time points were compared with their respective pre-transplant baseline.

6.3.2 IL-4 (figure 6.3.3)

The pre-rejection trend of changes in IL-4 gene expression in the rejectors mirrors quite closely to that seen in the non-rejectors over the same protocol time points. There was a significant suppression of peripheral IL-4 gene expression from the pre-transplant baseline at the early post-transplant time point in both rejectors and non-rejectors ($p=0.001$ for both groups). This change was followed by a significant rise in IL-4 gene expression at 1 week post-transplant ($p=0.039$ and 0.029 respectively), although this level of IL-4 was still significantly below the pre-transplant baseline in both groups ($p=0.009$ and 0.006 respectively).

The only difference demonstrated in the two groups is in the sequential change in IL-4 gene expression from 1 week post-transplant to 2 weeks post-transplant. While there was a significant rise in IL-4 level in non-rejectors over this two time points ($p=0.003$), no significant difference can be demonstrated in the rejectors over the same time period. Nevertheless, at the 2 weeks post-transplant time point, the level of IL-4 had returned to the pre-transplant baseline in both groups (p=NS when IL-4 levels at 2 weeks post-transplant time points in both groups were compared with their respective pre-transplant baselines).

6.3.3 IL-10 (figure 6.3.4)

Like in IL-4, the pre-rejection trend of changes in IL-10 gene expression in the rejectors also mirrors quite closely to that seen in the non-rejectors over the same protocol time points. In both rejectors and non-rejectors, there was a significant rise in peripheral IL-10 gene expression from the pre-transplant baseline at the early post-transplant time point ($p=0.004$ and 0.002 respectively). Although this change was followed by a significant fall at the 1 week post-transplant time point in the

Figure 6.3.3a - Rejectors

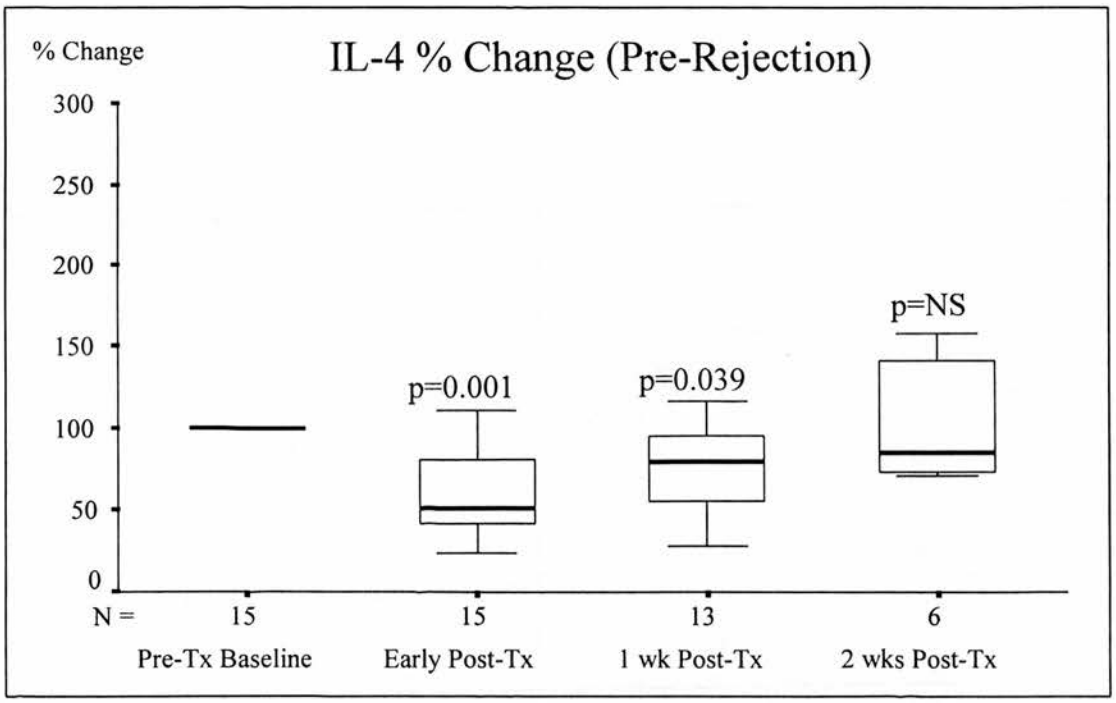


Figure 6.3.3b - Non-rejectors

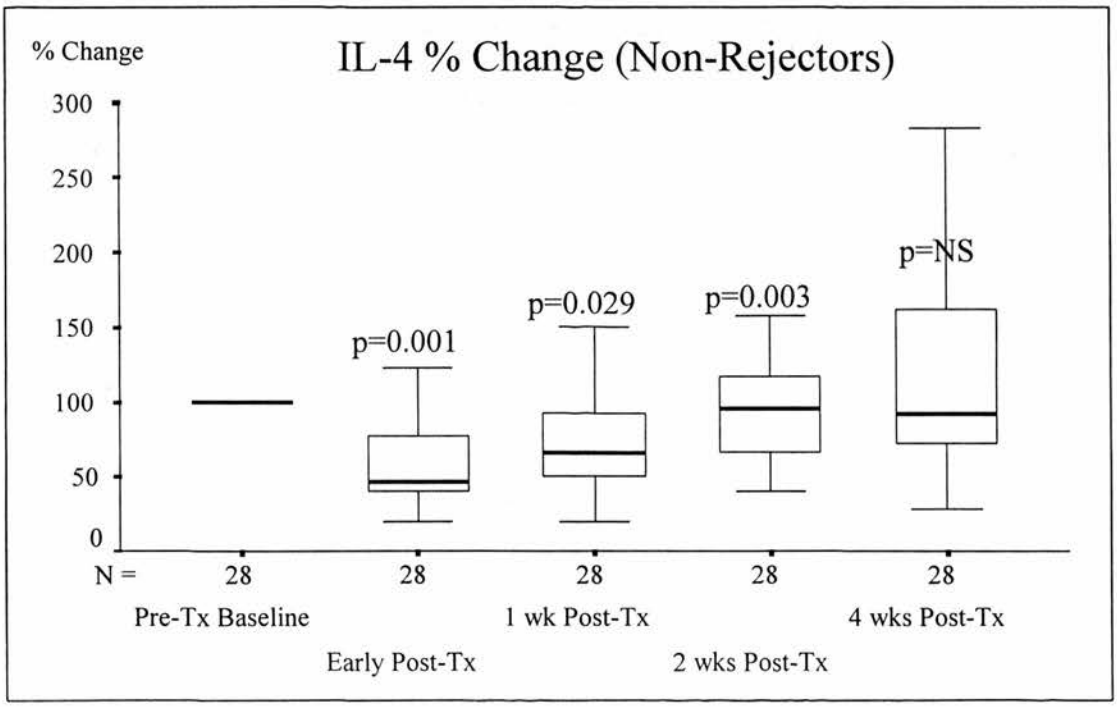


Figure 6.3.3 – Percentage change in IL-4 gene expression at pre-rejection time points in rejectors compared with non-rejectors over the same time points

rejectors (p=0.009) but no significant change over the same period in the non-rejectors (p=NS between early and 1 week post-transplant), the level of IL-10 gene expression at the 1 week post-transplant time point in both groups remained significantly higher than the pre-transplant baselines in both groups (p=0.028 and 0.008 respectively).

The major difference in the post-transplant trends in IL-10 gene expression in rejectors prior to the acute rejection episode and non-rejectors over the same time points was the level of IL-10 at the 2 weeks post-transplant time point. Although no significant change in IL-10 expression was seen between the 1 week and 2 weeks post-transplant time points in both groups of patients, the level of IL-10 at 2 weeks post-transplant was not significantly different from the pre-transplant baseline in the rejectors but in the non-rejectors, IL-10 remained significantly above the pre-transplant baseline at the 2 weeks post-transplant time point (p=0.041).

6.3.4 IL-5 (figure 6.3.5)

There are differences in the post-transplant level of IL-5 gene expression between rejectors (pre-rejection time points) and non-rejectors despite the apparent similarities between the two groups in their sequential pattern of changes in IL-5 expression. There was a significant fall in IL-5 at the early post-transplant time point in both rejectors (p=0.041) and non-rejectors (p=0.012), but no further significant sequential changes were seen in either group from early to 2 weeks post-transplant.

However, the level of IL-5 at the 1 and 2 weeks post-transplant time points were not significantly different from the pre-transplant baseline in the rejectors, while in the non-rejectors, IL-5 expression remained significantly below the pre-transplant baseline at the 1 week (p=0.001) and 2 weeks (p=0.001) post-transplant time points.

Figure 6.3.4a - Rejectors

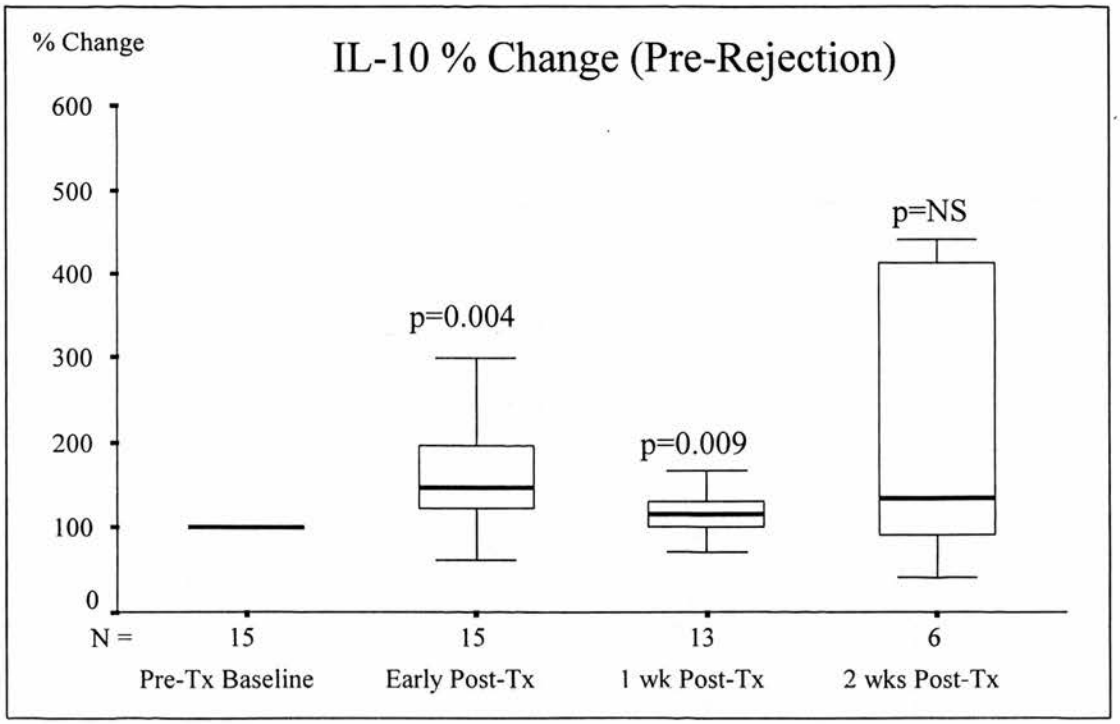


Figure 6.3.4b - Non-rejectors

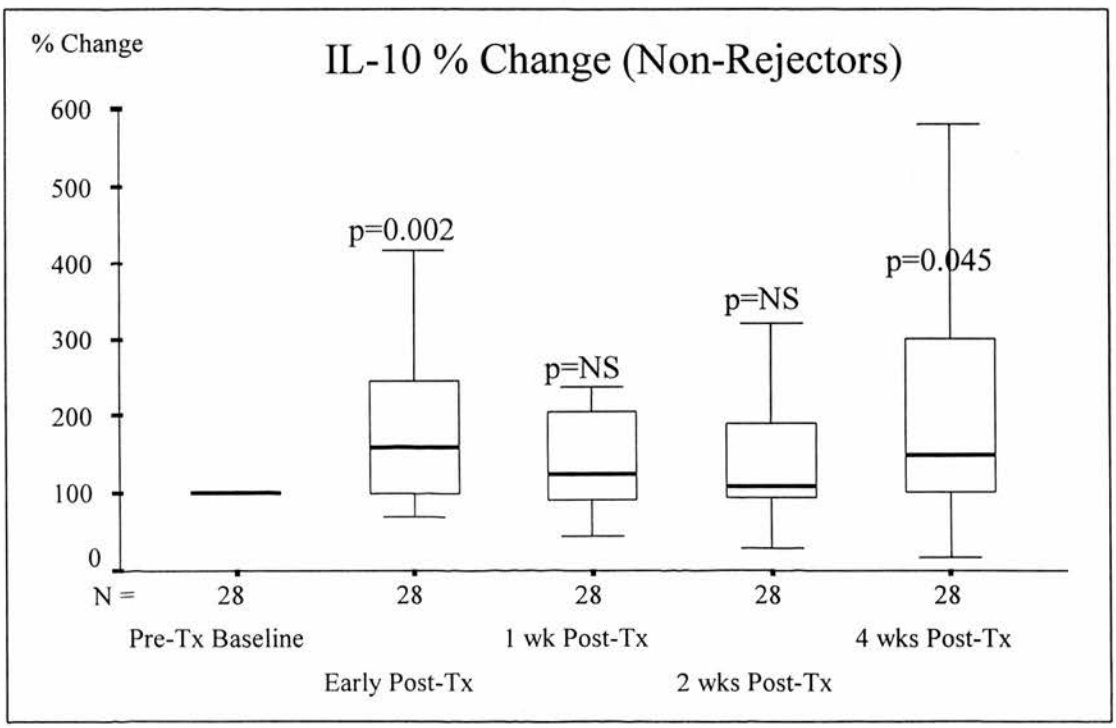


Figure 6.3.4 – Percentage change in IL-10 gene expression at pre-rejection time points in rejectors compared with non-rejectors over the same time points

Figure 6.3.5a - Rejectors

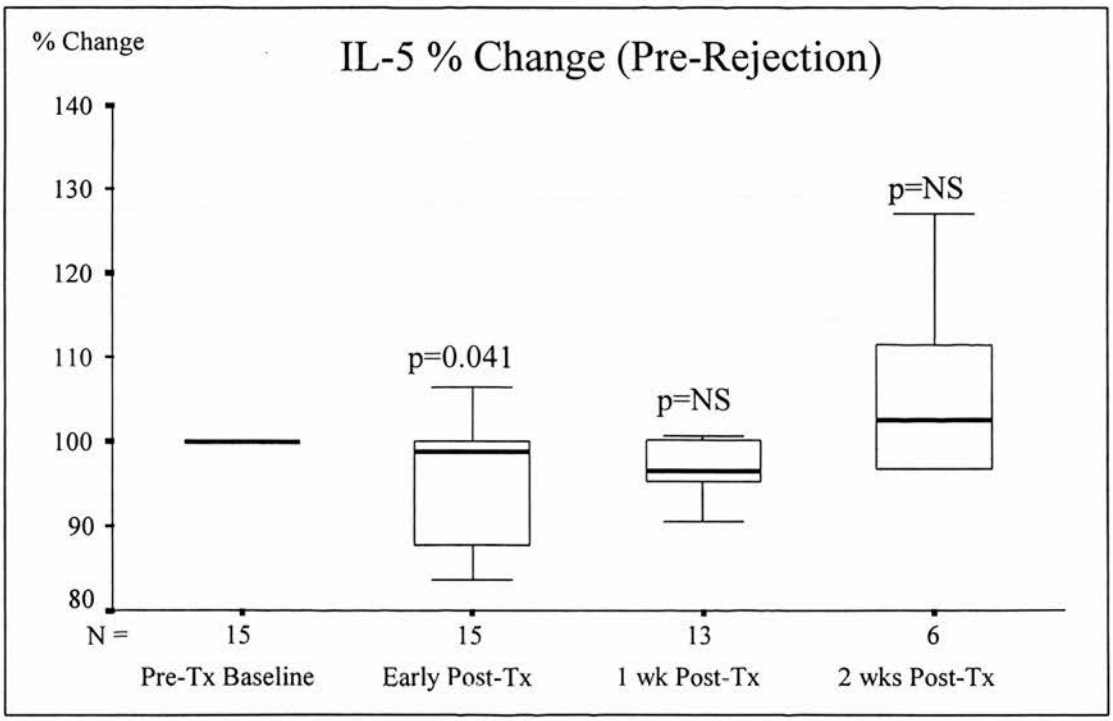


Figure 6.3.5b - Non-rejectors

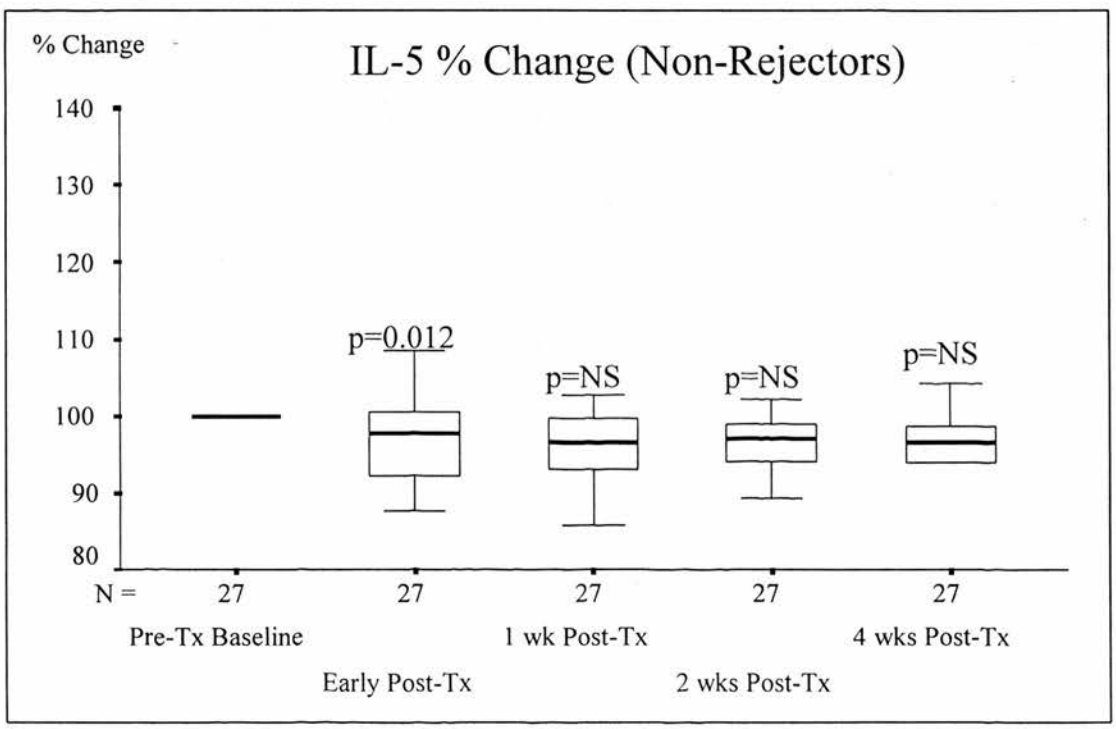


Figure 6.3.5 – Percentage change in IL-5 gene expression at pre-rejection time points in rejectors compared with non-rejectors over the same time points

6.3.5 IL-13 (figure 6.3.6)

The IL-13 gene expression profile in the non-rejectors, like the profiles for IL-2 and IFN-γ, was rather "flat" with no significant differences either sequentially from one time point to the next or when all post-transplant time points were compared with their respective pre-transplant baseline.

In the rejectors, there was no significant change in IL-13 gene expression at the early post-transplant time point either. Although there was a significant increase in IL-13 at 1 week post-transplant ($p=0.011$) from the early post-transplant time point, this level was nevertheless not significantly different from the pre-transplant baseline. Interestingly, the level of IL-13 at 2 weeks post-transplant was significantly higher than the pre-transplant baseline ($p=0.028$) even though this level was not significantly different from the previous time point at 1 week post-transplant.

6.3.6 GrB (figure 6.3.7)

There are interesting differences in GrB gene expression between rejectors (pre-rejection profile) and non-rejectors. While there was a significant decrease in GrB at the early post-transplant time point in the rejectors ($p=0.001$), no significant change was demonstrated in the non-rejectors. There were no further significant sequential changes in GrB expression at all the subsequent time points in either groups, and the level of GrB at the 1 week post-transplant time point was significantly below the pre-transplant baseline in both rejectors ($p=0.006$) and non-rejectors ($p=0.003$).

However, by 2 weeks post-transplant, the level of GrB in the rejectors was no longer significantly different from the pre-transplant baseline level, while in the non-rejectors, GrB level remained significantly below the pre-transplant baseline at the 2 weeks post-transplant time point ($p=0.011$).

Figure 6.3.6a - Rejectors

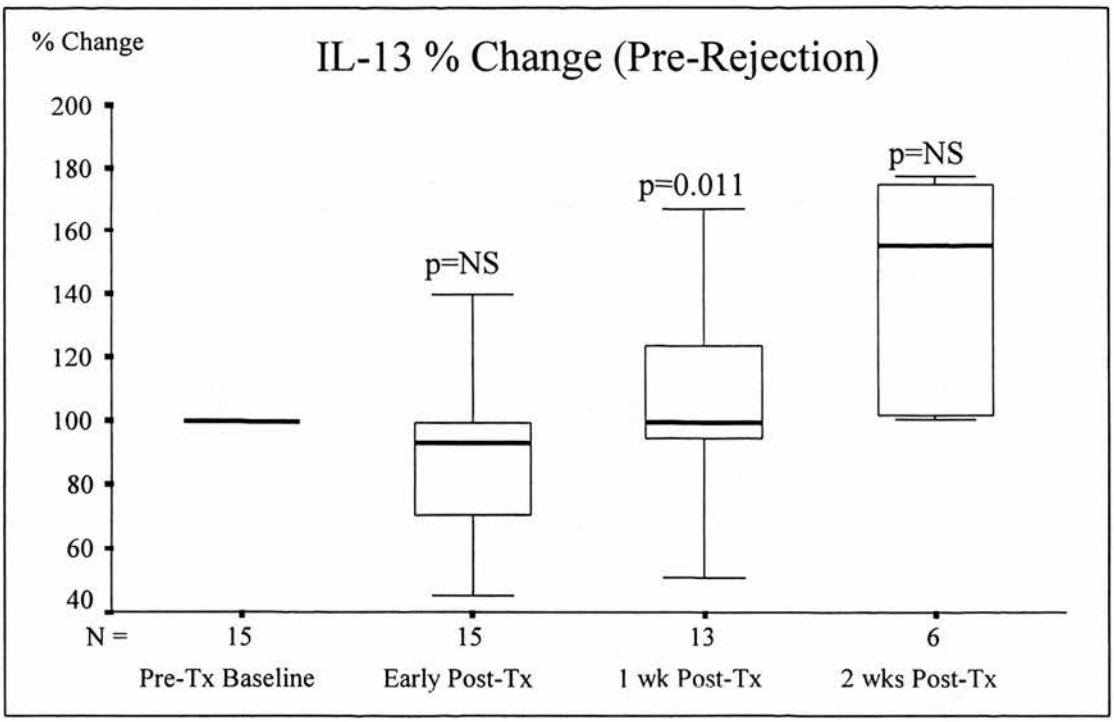


Figure 6.3.6b - Non-rejectors

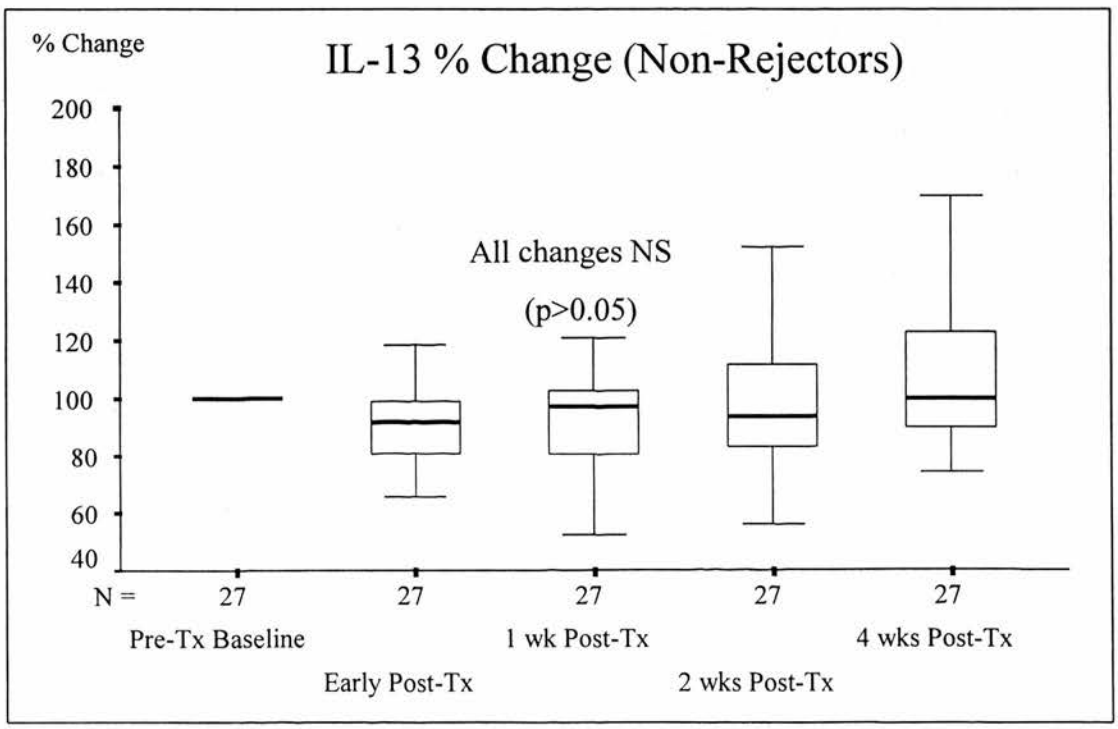


Figure 6.3.6 – Percentage change in IL-13 gene expression at pre-rejection time points in rejectors compared with non-rejectors over the same time points

Figure 6.3.7a - Rejectors

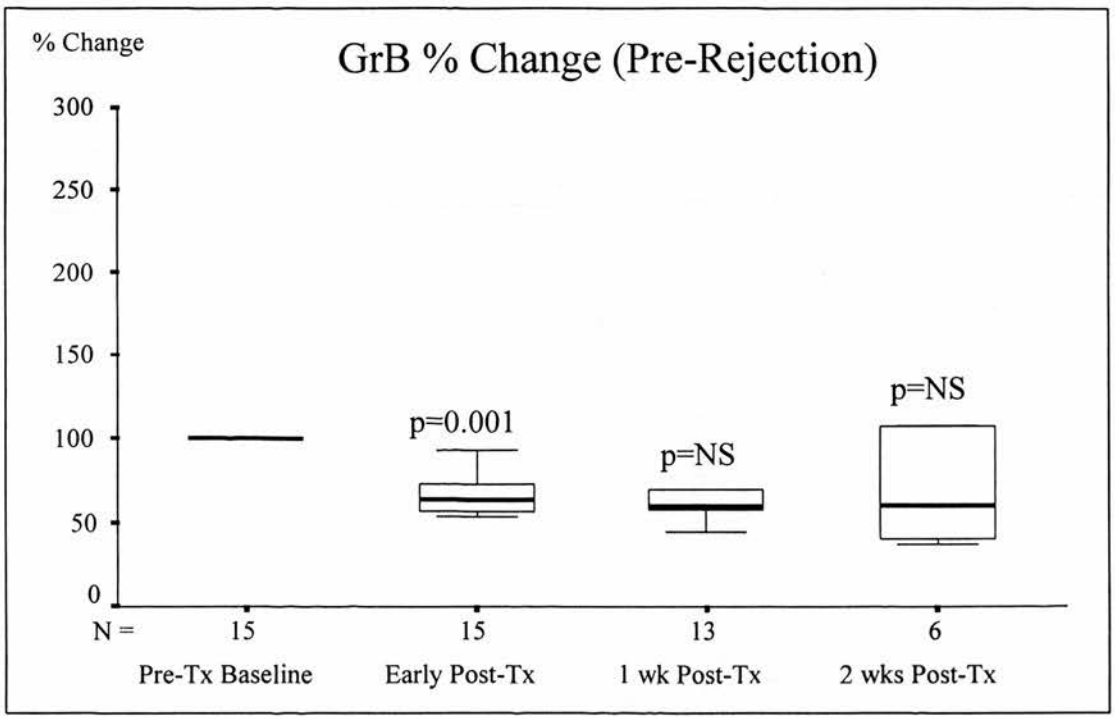


Figure 6.3.7b - Non-rejectors

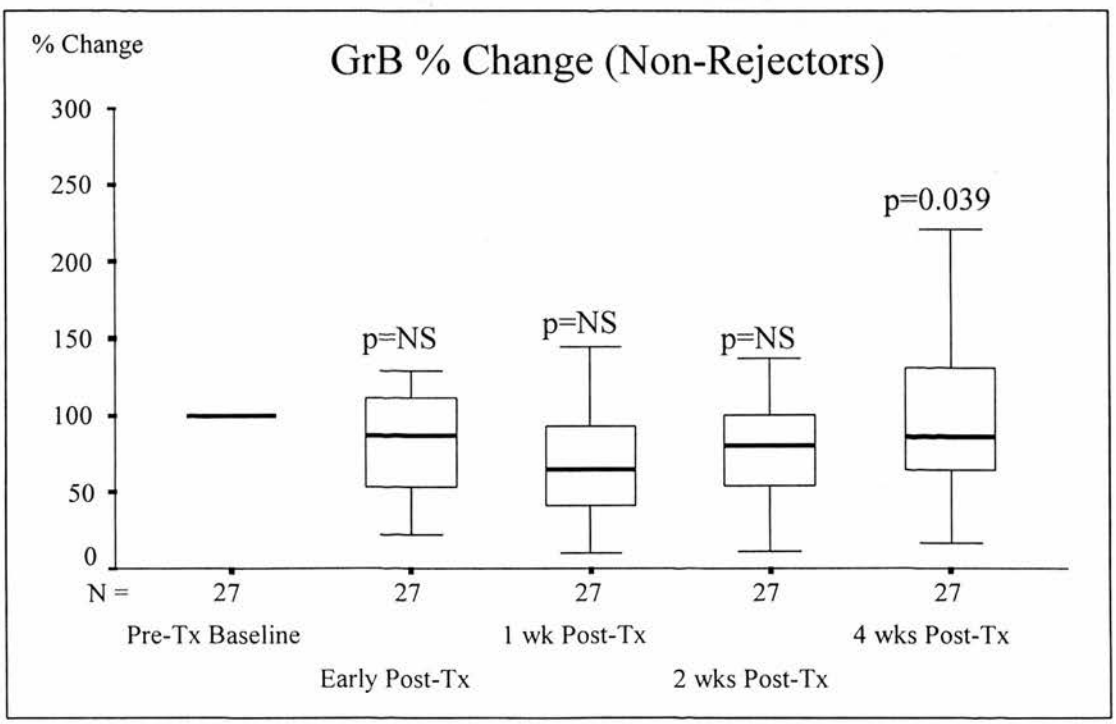


Figure 6.3.7 – Percentage change in GrB gene expression at pre-rejection time points in rejectors compared with non-rejectors over the same time points

6.3.7 FasL (figure 6.3.8)

The post-transplant profiles of FasL in rejectors and non-rejectors are quite similar. There were no significant sequential changes in FasL gene expression in the first 4 protocol time points in both groups of patients. The level of FasL at 1 week post-transplant in both groups were significantly below their respective pre-transplant baselines ($p=0.019$ in rejectors and $p=0.032$ in non-rejectors). However, FasL level at 2 weeks post-transplant remained significantly below the pre-transplant baseline in the rejectors ($p=0.028$), while no significant difference between the level of FasL at 2 weeks post-transplant and the pre-transplant baseline was demonstrated in the non-rejectors.

6.3.8 IL-10/IL-4 Ratio (figure 6.3.9)

The pre-rejection IL-10/IL-4 profile in rejectors over the first 4 protocol time points is similar to the profile for non-rejectors over the same time points. In both rejectors and non-rejectors, there was a significant rise in IL-10/IL-4 ratio at the early post-transplant time point ($p=0.001$ and <0.001 respectively), followed by a significant fall at 1 week post-transplant ($p=0.006$ and 0.003 respectively) to a level that remained significantly above the pre-transplant baselines ($p=0.005$ and <0.001 respectively).

In the rejectors, although the IL-10/IL-4 ratio at 2 weeks post-transplant was not significantly different from the ratio at 1 week post-transplant, this ratio had nevertheless returned back to the pre-transplant baseline level ($p=$NS when the 2 weeks post-transplant ratio was compared with the pre-transplant baseline ratio). On the other hand, despite a further significant sequential fall in IL-10/IL-4 ratio in the non-rejectors at the 2 weeks post-transplant time point, this ratio remained significantly above the pre-transplant baseline ($p=0.038$).

Figure 6.3.8a - Rejectors

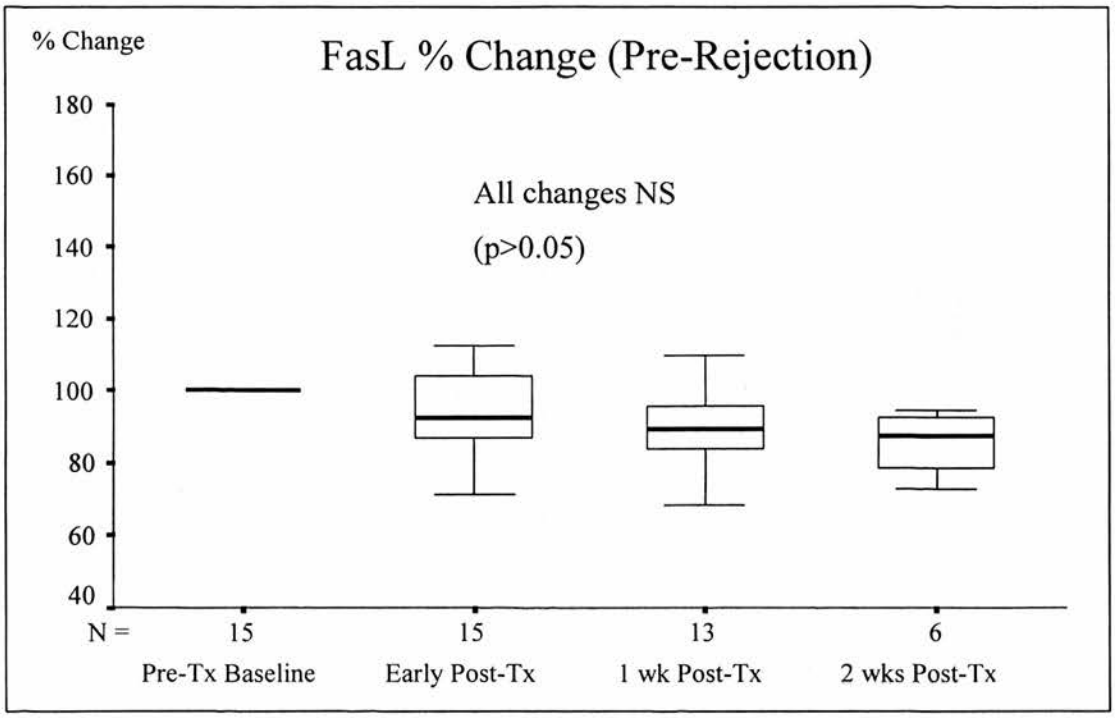


Figure 6.3.8b - Non-rejectors

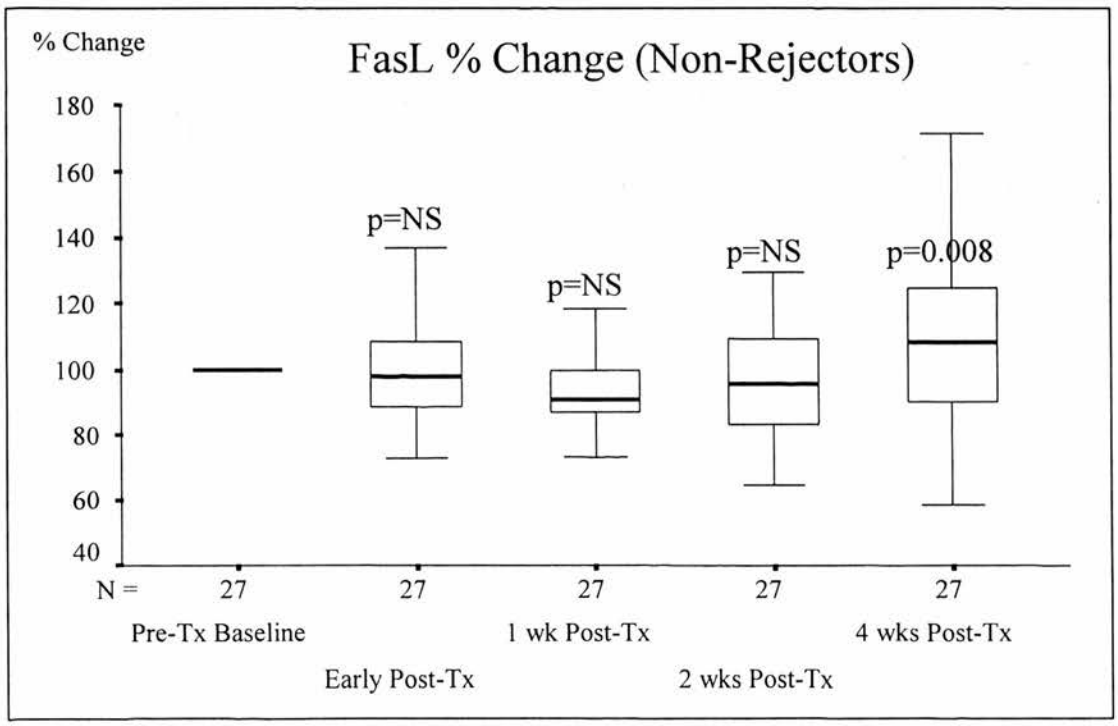


Figure 6.3.8 – Percentage change in FasL gene expression at pre-rejection time points in rejectors compared with non-rejectors over the same time points

Figure 6.3.9a - Rejectors

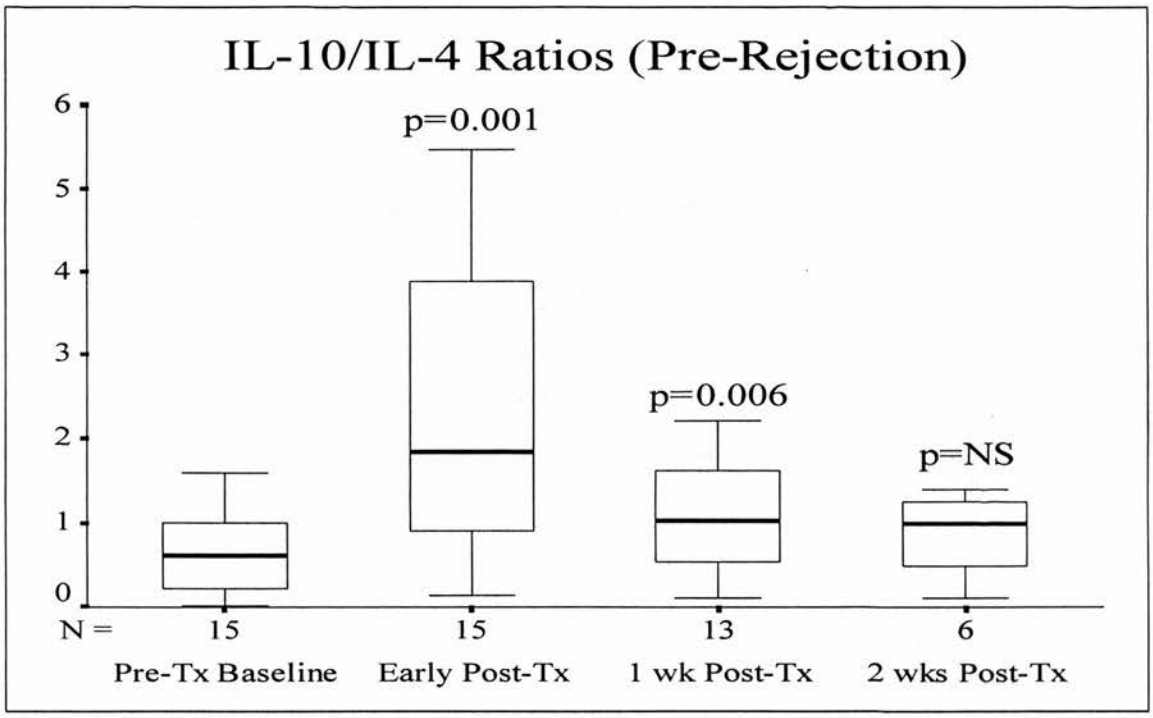


Figure 6.3.9b - Non-rejectors

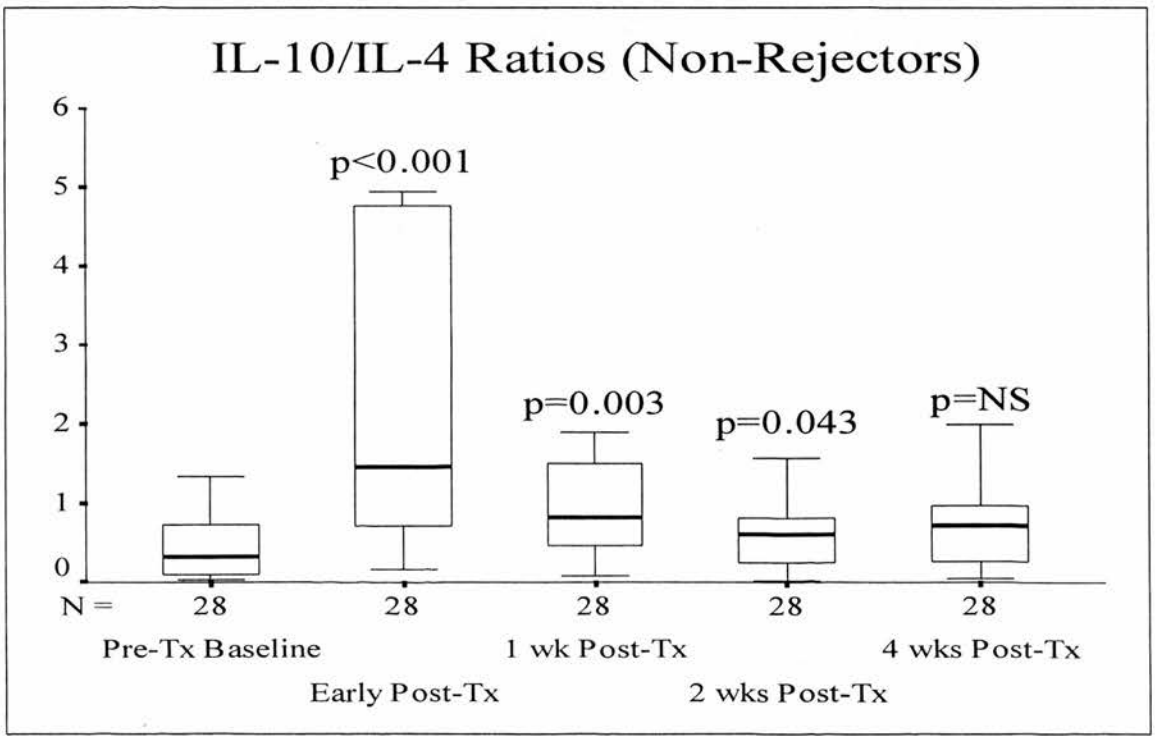


Figure 6.3.9 – IL-10/IL-4 ratios at pre-rejection time points in rejectors compared with non-rejectors over the same time points

6.3.9 IL-4/IL-2 Ratio (figure 6.3.10)

There was a significant fall in IL-4/IL-2 ratio at the early post-transplant time point ($p=0.011$) in the non-rejectors which was not seen in the rejectors. In both groups of patients, there was a significant subsequent rise in the ratio at 1 week post-transplant ($p=0.046$ for rejectors and 0.036 for non-rejectors), but the ratios at this time point were not significantly different from their respective pre-transplant baselines in the two groups. IL-4/IL-2 ratio remained at the pre-transplant baseline level from 1 to 2 weeks post-transplant in both groups.

6.3.10 IL-4/IFN-γ Ratio (figure 6.3.11)

The pre-rejection IL-4/IFN-γ profile in rejectors is similar to the profile seen in non-rejectors over the same protocol time points.

There was a significant fall in IL-4/IFN-γ ratio at the early post-transplant time point in both rejectors and non-rejectors ($p=0.003$ and 0.007 respectively), which was followed by a significant rise in the ratio at 1 week post-transplant ($p=0.002$ and 0.003 respectively) back to the pre-transplant baseline level (p=NS when 1 week post-transplant ratios were compared with the pre-transplant ratios).

The only difference between rejectors and non-rejectors is seen at 2 weeks post-transplant. Although there were no significant changes in IL-4/IFN-γ ratio from 1 to 2 weeks post-transplant in both groups, IL-4/IFN-γ ratio at 2 weeks post-transplant in rejectors was significantly higher than the pre-transplant baseline ($p=0.028$), but no significant difference was demonstrated in the same comparison in the non-rejectors.

Figure 6.3.10a - Rejectors

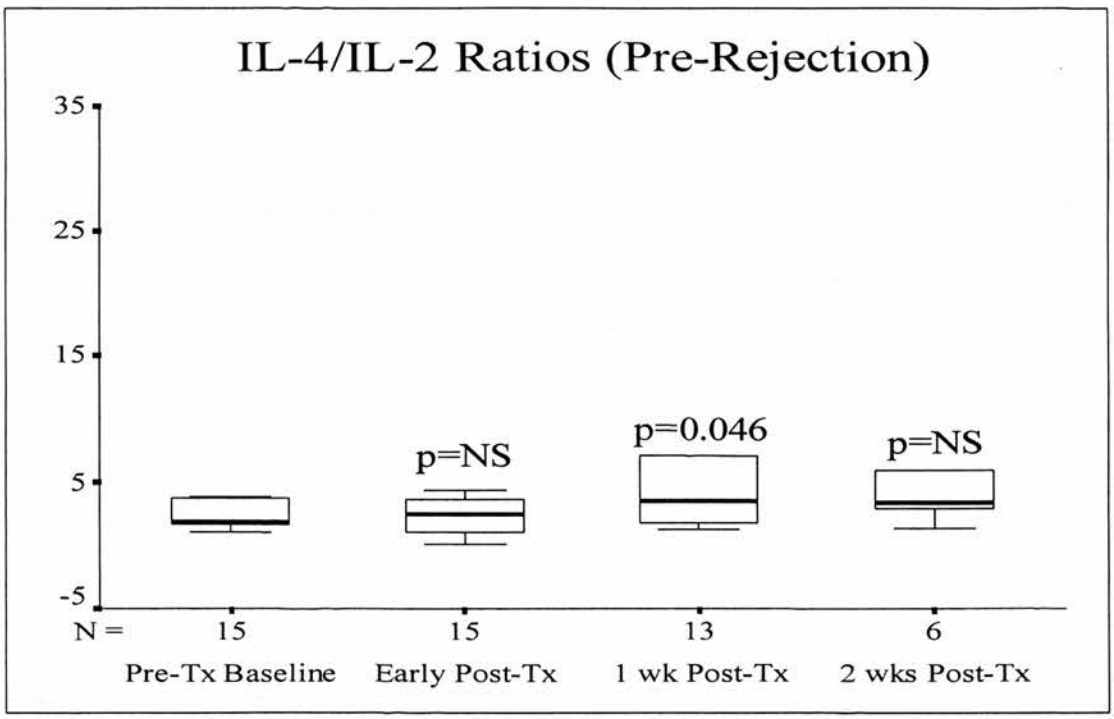


Figure 6.3.10b - Non-rejectors

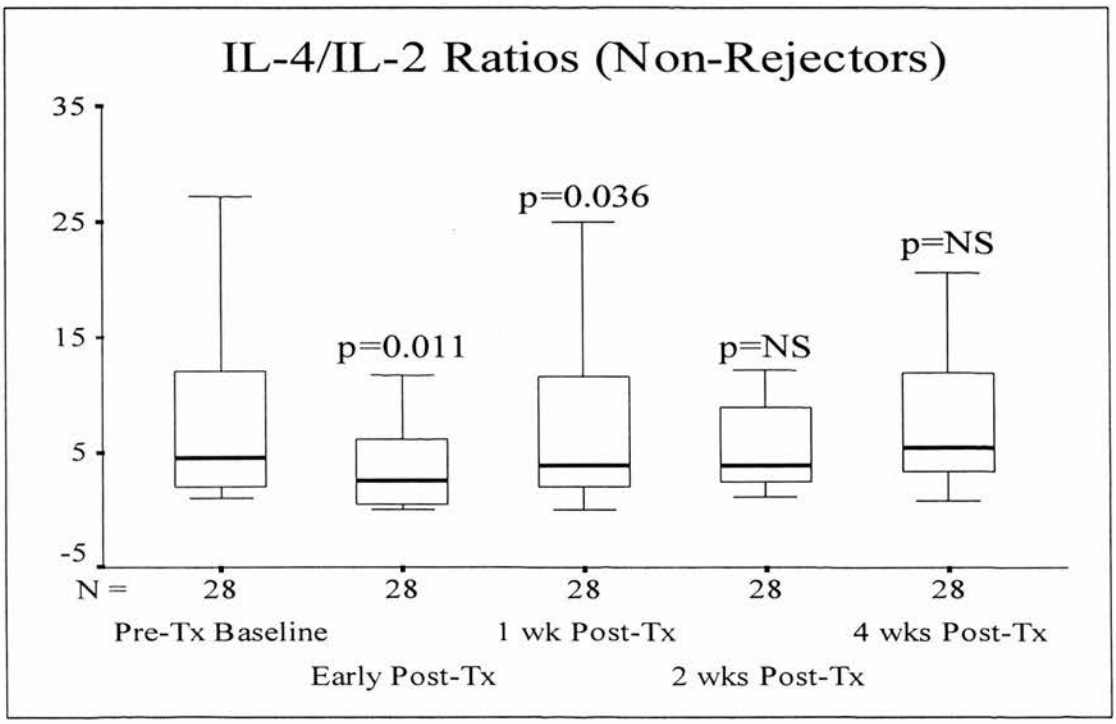


Figure 6.3.10 – IL-4/IL-2 ratios at pre-rejection time points in rejectors compared with non-rejectors over the same time points

Figure 6.3.11a - Rejectors

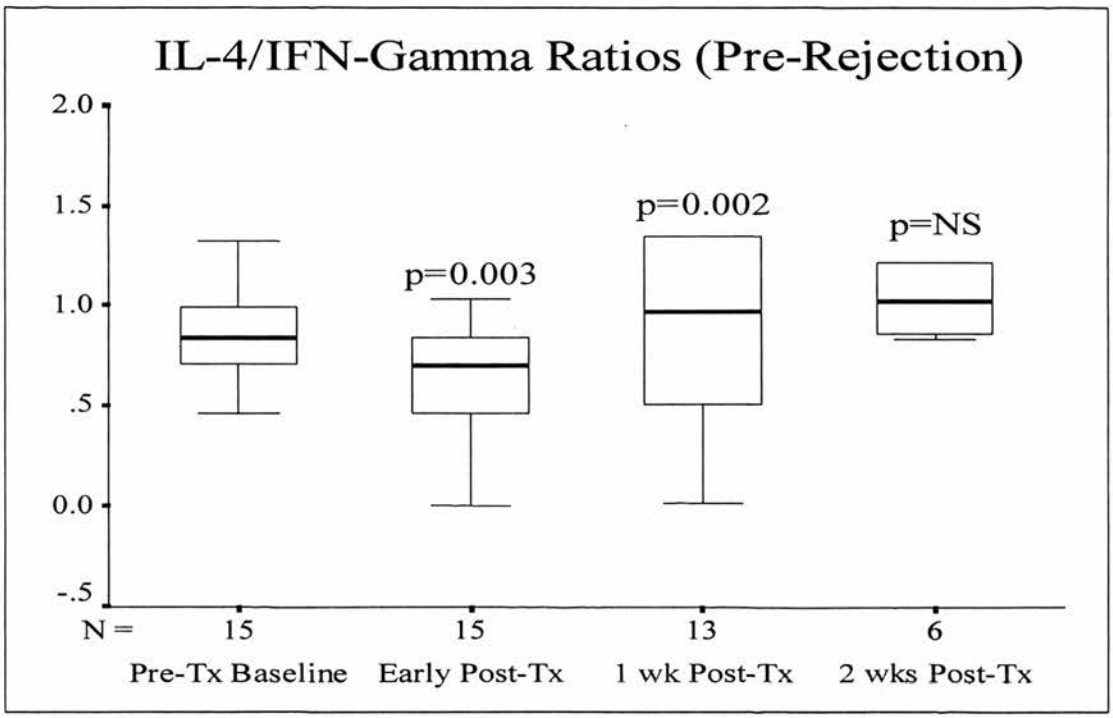


Figure 6.3.11b - Non-rejectors

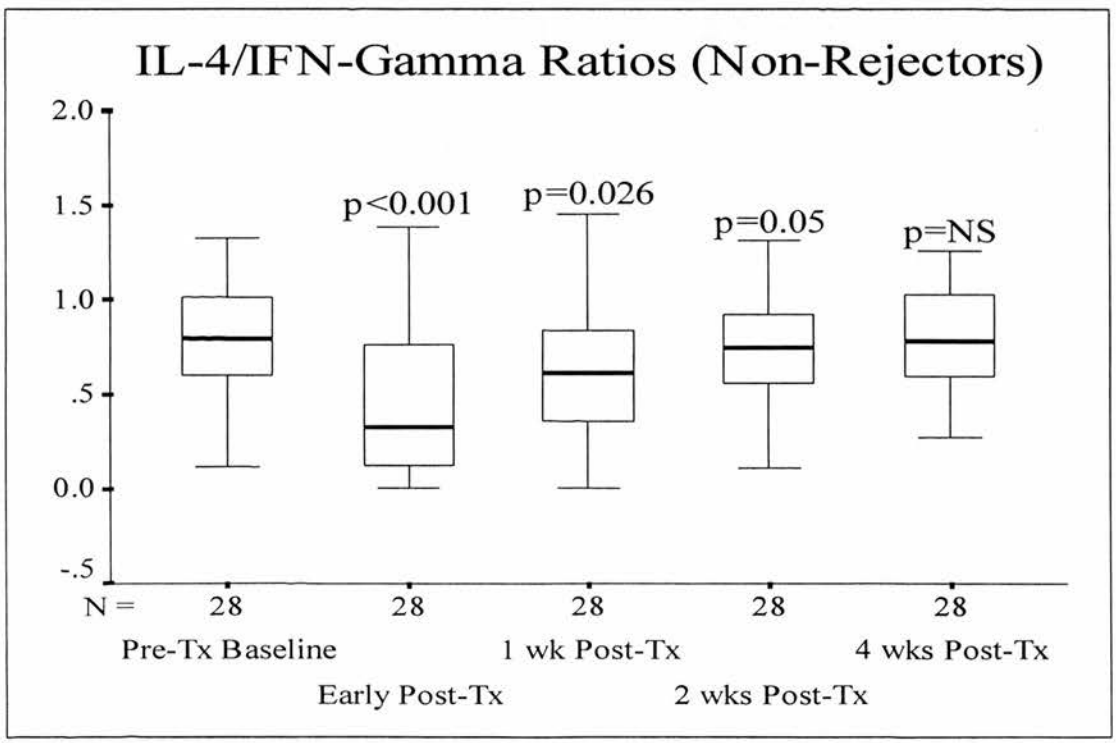


Figure 6.3.11 – IL-4/IFN-γ ratios at pre-rejection time points in rejectors compared with non-rejectors over the same time points

6.3.11 IL-10/IL-2 and IL-10/IFN-γ Ratios (figures 6.3.12 and 6.3.13)

The post-transplant profiles of IL-10/IL-2 and IL-10/IFN-γ ratios are similar in rejectors (pre-rejection) and non-rejectors. In both groups, a highly significant increase in the IL-10/IL-2 ($p=0.001$ in both groups) and IL-10/IFN-γ ($p=0.002$ and 0.001 respectively) ratios were seen at the early post-transplant time point, followed by no further significant sequential changes in the remaining protocol time points. Both ratios at the 1 week post-transplant time point remained significantly above their respective pre-transplant baselines in rejectors and non-rejectors ($p=0.003$ and <0.001 respectively for IL-10/IL-2, $p=0.004$ and 0.006 respectively for IL-10/IFN-γ).

At the 2 weeks post-transplant time point, only the IL-10/IL-2 ratio in the rejectors remained significantly above its pre-transplant baseline ($p=0.028$). No significant differences were demonstrated when IL-10/IL-2 ratio in non-rejectors and IL-10/IFN-γ ratio in both rejectors and non-rejectors at the 2 weeks post-transplant time point were compared with their respective pre-transplant baselines.

6.3.12 IFN-γ/IL-2 Ratio (figure 6.3.14)

IFN-γ/IL-2 profiles for rejectors (pre-rejection time points) and non-rejectors are very similar. In both groups of patients, no significant differences were demonstrated when the IFN-γ/IL-2 ratios at each time point were compared sequentially with each other or when all post-transplant time points were compared with the pre-transplant baseline.

Figure 6.3.12a - Rejectors

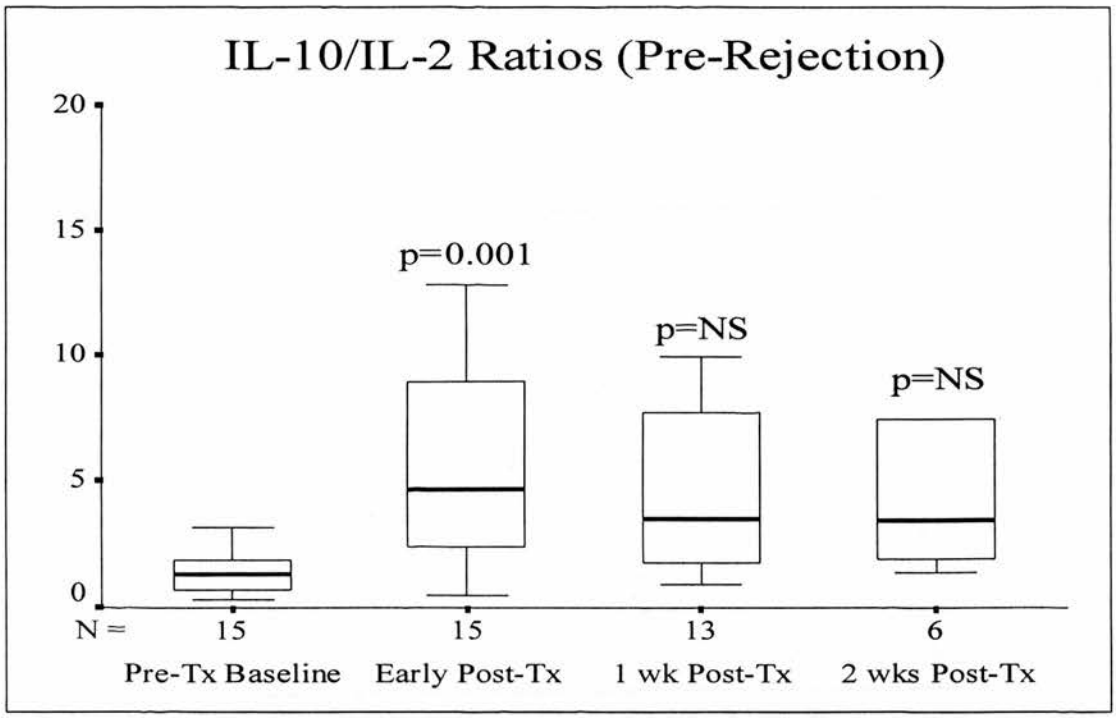


Figure 6.3.12b - Non-rejectors

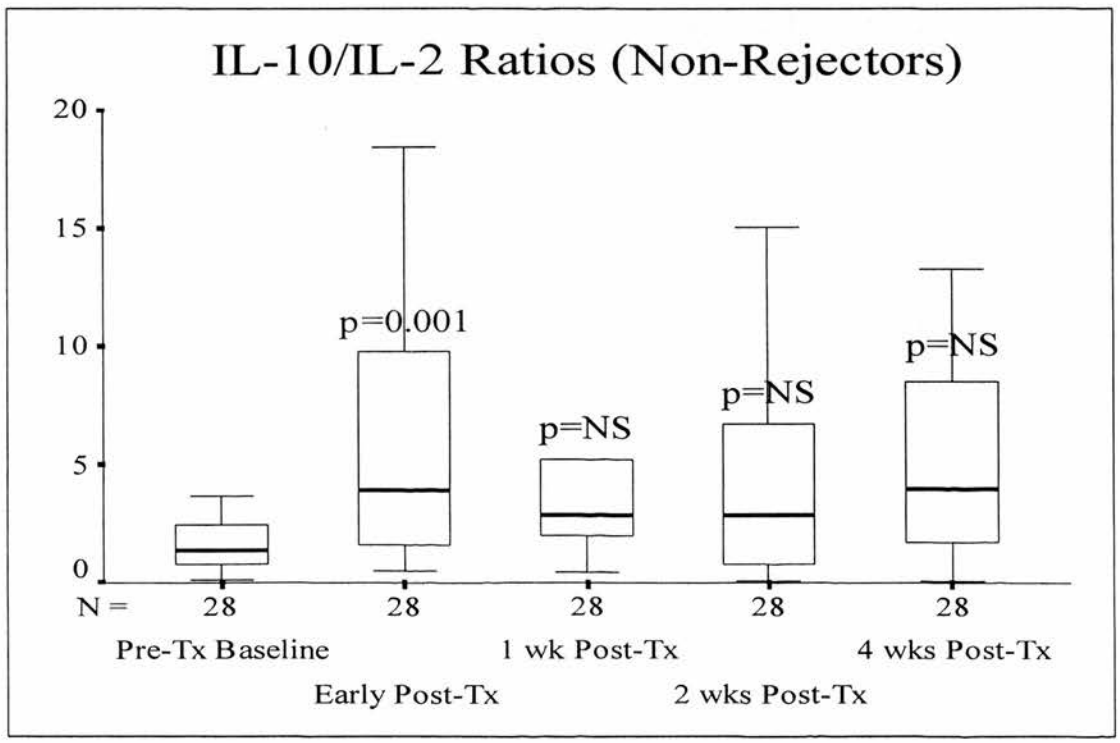


Figure 6.3.12 – IL-10/IL-2 ratios at pre-rejection time points in rejectors compared with non-rejectors over the same time points

Figure 6.3.13a - Rejectors

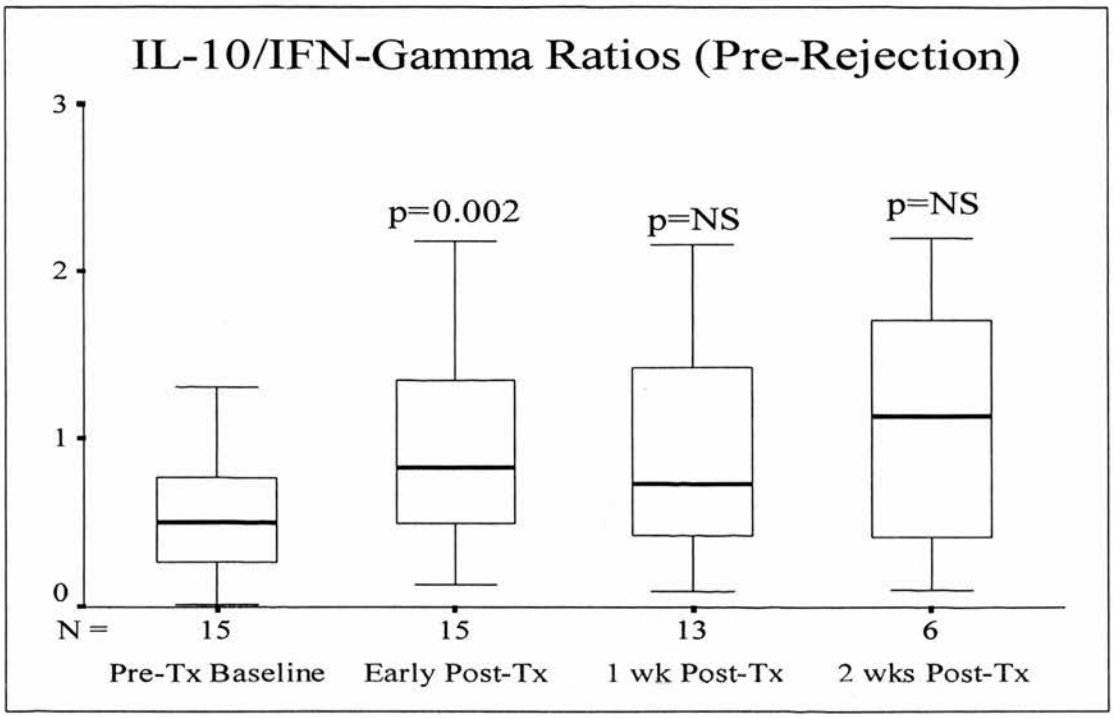


Figure 6.3.13b - Non-rejectors

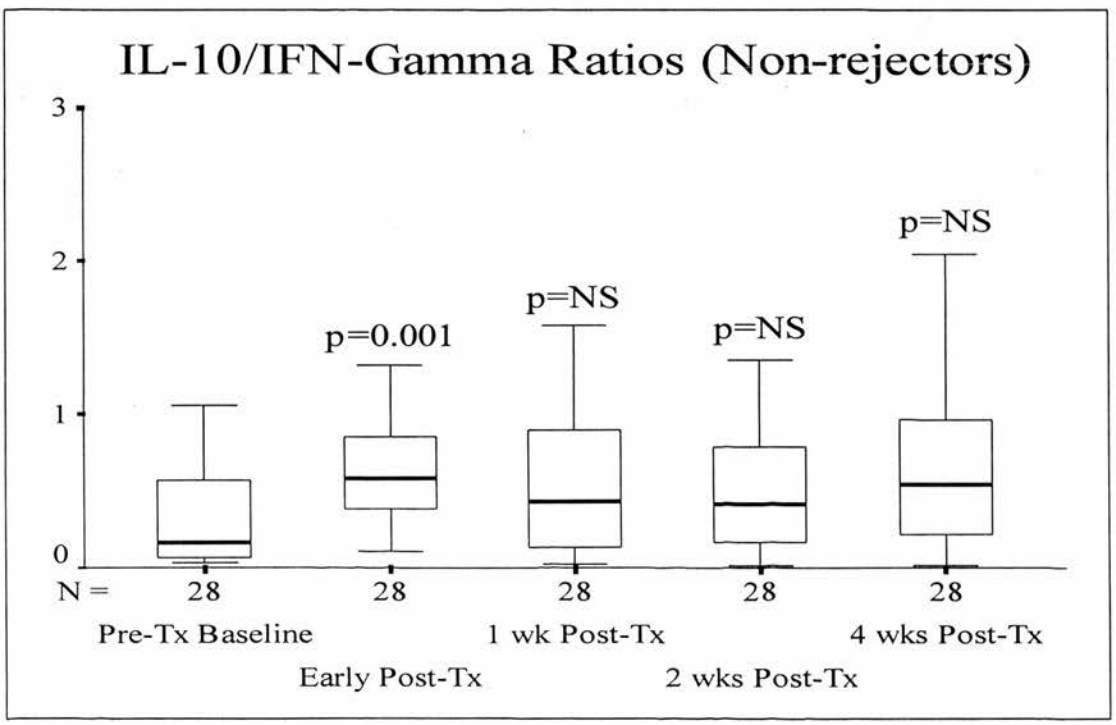


Figure 6.3.13 – IL-10/IFN-γ ratios at pre-rejection time points in rejectors compared with non-rejectors over the same time points

Figure 6.3.14a - Rejectors

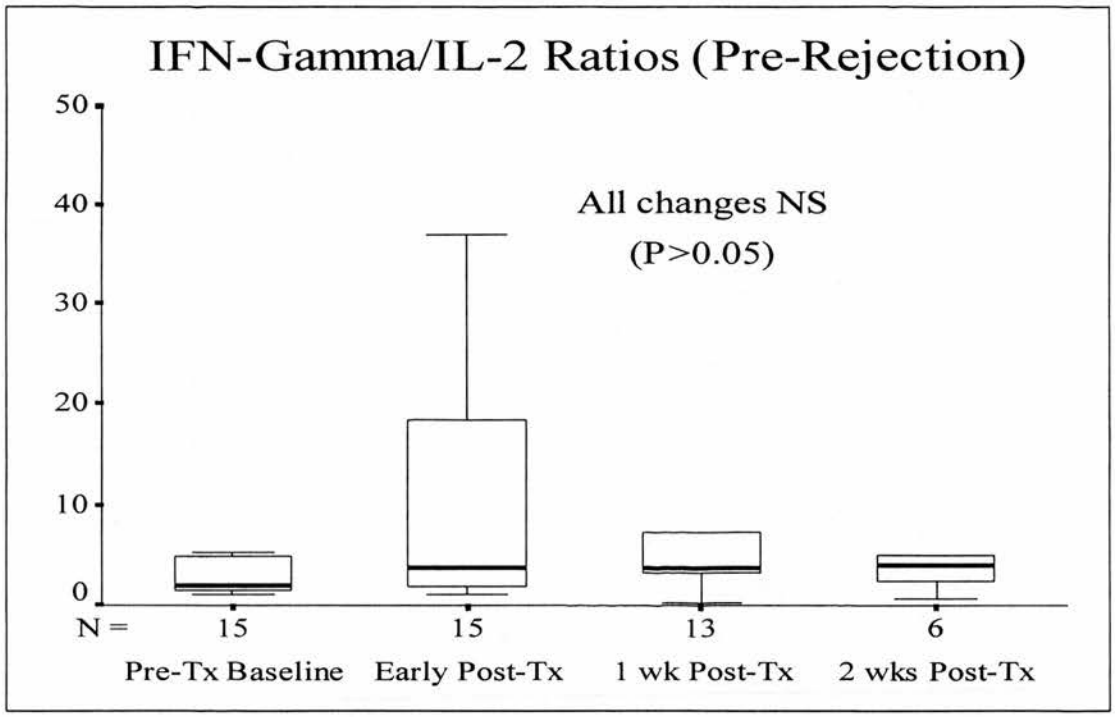


Figure 6.3.14b - Non-rejectors

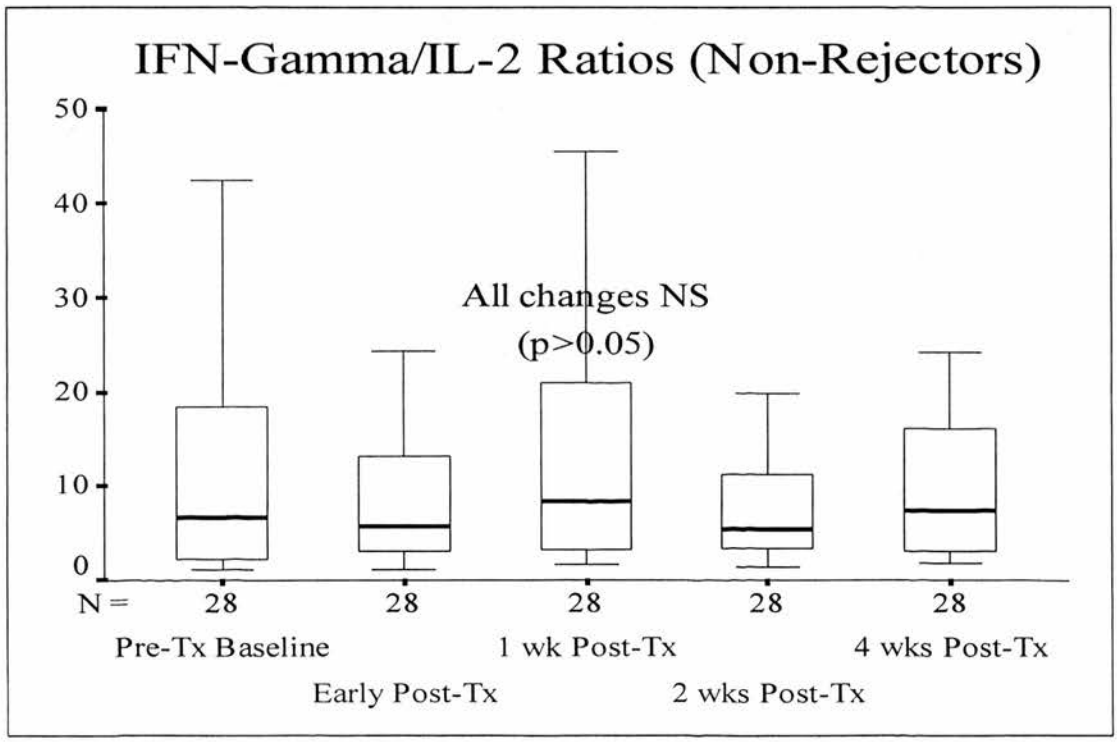


Figure 6.3.14 – IFN-γ/IL-2 ratios at pre-rejection time points in rejectors compared with non-rejectors over the same time points

6.4 Comparison between rejectors and non-rejectors at each protocol time point

This analysis of the data was undertaken to see if the differences in rejectors' and non-rejectors' profiles shown by the sequential analyses in the previous sections are also demonstrable when RT-PCR ELISA data of the two cohorts of patients at each time point are compared, accepting the limitations of semi-quantitative data.

The non-parametric Mann-Whitney U test was applied to all the data from the first 4 protocol sampling time points in the rejectors (prior to the acute rejection episode) and non-rejectors. This test was chosen to enable a statistical comparison of the percentage changes in the level of cytokine/CTL activation marker gene expression at each protocol sampling time point following renal transplantation between the rejectors and the non-rejectors. In the same manner, the Mann-Whitney U test was also applied to compare the ratios of the cytokine gene expression (IL-10/IL-4, IL-4/IL-2, IL-4/IFN-γ, IL-10/IL-2, IL-10/ IFN-γ and IFN-γ/IL-2) at each protocol sampling time point between the rejectors and non-rejectors. The results of both Mann-Whitney U tests are set out in tables 6.4.1 and 6.4.2 respectively. These analyses are valid because only data from the same time point in the two groups were compared.

In the table 6.4.1, "-" indicates that the statistical comparison between the two groups of patients was not performed at the pre-transplant time point. The reason for this is that this time point was the baseline from which all the other sequential changes in cytokines/CTL activation markers gene expression were compared with, and since it had been arbitrarily set at 100%, statistical comparison between the groups at this time point would therefore be meaningless. Comparisons at each time point between the rejectors and non-rejectors which were not statistically significant (i.e. p value greater than 0.05) is marked with "NS" and for comparisons which were statistically significant, a 2-tailed p value is quoted.

Table 6.4.1 – Mann-Whitney U test of percentage change in cytokine/CTL activation marker gene expression between rejectors and non-rejectors.

		Protocol sampling time points			
		Pre-Tx	Early Post-Tx	1 wk Post-Tx	2 wks Post-Tx
Cytokine and CTL gene expression % change	IL-2	-	<0.001	<0.001	0.013
	IFN-γ	-	NS	NS	NS
	IL-4	-	NS	NS	NS
	IL-10	-	NS	NS	NS
	IL-5	-	NS	NS	NS
	IL-13	-	NS	NS	0.009
	GrB	-	NS	NS	NS
	FasL	-	NS	NS	NS

Table 6.4.1 – Mann-Whitney U test of cytokine gene expression ratios between rejectors and non-rejectors.

		Protocol sampling time points			
		Pre-Tx	Early Post-Tx	1 wk Post-Tx	2 wks Post-Tx
Cytokine gene expression ratios	IL-10/IL-4	NS	NS	NS	NS
	IL-4/IL-2	NS	NS	NS	NS
	IL-4/IFN-γ	NS	NS	0.047	0.034
	IL-10/IL-2	NS	NS	NS	NS
	IL-10/IFN-γ	NS	NS	NS	NS
	IFN-γ/IL-2	NS	NS	NS	NS

Chapter 7: DISCUSSION OF THE GENE EXPRESSION PROFILES OF PERIPHERAL T CELLS

In this chapter, the significance and interpretation of the sequential profile of changes in gene expression of the individual cytokines/CTL activation markers are discussed in the first section while the relationships between the changes in the level of the individual cytokine/CTL activation marker at each sampling time point is discussed in the next section. The relevance of the changes seen in the profiles for the cytokine ratios is discussed in the penultimate section, and the final section discusses the statistical comparisons of the rejectors versus non-rejectors data at each protocol sampling time points prior to acute rejection. All the relevant sub-sections referred to from the previous chapters will be referenced by their sub-section numbers within square brackets.

7.1 Sequential changes in cytokine/CTL activation marker gene expression

In this section, the sequential pattern of changes in peripheral T cell gene expression of the study cohort will be discussed individually for each cytokine/CTL activation marker studied in the project. The cytokines and CTL activation markers will be discussed in the same order as presented in the previous chapter.

7.1.1 IL-2 and IFN-γ

The presence or an increase in proinflammatory cytokines like IL-2 and IFN-γ at the time of an acute allograft rejection process is theoretically sound and logical, and indeed these two cytokines have been found to be associated with acute rejection in several studies [Vie et al, 1985; Simpson et al, 1989; Colvin et al, 1989; Dallman et al, 1991a; Dallman et al, 1991c; Papp et al, 1992; Takeuchi et al, 1992; Wu et al,

1992; Papp et al, 1992; Takeuchi et al, 1992; Wu et al, 1992; O'Connell et al, 1993; Kutukculer et al, 1995; Gaweco et al, 1995], however other studies have not been able to confirm this association (as reviewed in sub-sections 1.5.1 and 1.5.2). Our study also did not reveal an increase in IL-2 or IFN-γ gene expression with acute rejection, but instead found a surprising early reduction in the expression of peripheral T cell IL-2 and IFN-γ transcripts in patients who subsequently go on to experience acute rejection. The return of IL-2 and IFN-γ gene expression at 1 week and 4 weeks following anti-rejection therapy back to the pre-transplant baseline as in the non-rejectors' profiles, suggests that the early reduction in the expression of both cytokines demonstrated by our study was a true reflection of the changes in alloreactivity of Th1 cells following their exposure to the renal allograft.

It may be that the reduction in the level of peripheral blood gene expression of both Th1 cytokines in the first week following renal transplantation is due to the sequestration of this subset of T helper cells within the allograft or other lymphoid compartments such as bone marrow or spleen in patients who then subsequently go on to experience acute rejection. This sequestration of Th1 producing cells within the renal allograft early on following transplantation may be the source of the cytokine trigger for the subsequent acute rejection process.

The reduction of both Th1 cytokines at the time of rejection (marked by the pre-anti-rejection therapy time point) or shortly following completion of anti-rejection therapy (the early post-anti-rejection therapy time point) may again be a reflection of further sequestration of Th1 cytokine-producing cells in the allograft or lymphoid compartments during the acute phase of the rejection process. Similar findings of a decrease in induced IL-2 gene expression by peripheral mononuclear cells below the pre-transplant level at the time of acute rejection in clinical renal transplantation as well as in a canine model of renal transplantation have been reported by one group of workers [Zucker et al, 1996].

7.1.2 IL-4

Of all the cytokines and CTL activation markers studied in the project, peripheral IL-4 gene expression profiles showed the most dramatic of changes in both rejectors and non-rejectors by having statistically significant sequential changes at almost all the time points [6.1.3 and 6.3.2]. Overall, the profile of changes in peripheral IL-4 gene expression when correlated with the clinical course of our study cohort suggests it to be a cytokine which is highly sensitive to the effect of immunosuppressive drugs.

The highly significant fall in peripheral IL-4 gene expression in both groups of patients following renal transplantation in the early post-transplant period prior to a steady return back to the pre-transplant baseline expression level at 2 weeks post-transplant [6.3.2] may be a reflection of the potent loading dose effect on the level of peripheral IL-4 gene expression of the immunosuppressive drugs. Although one could equally suggest that these early fall in the level of peripheral IL-4 gene expression in both groups of patients may be the immunological consequence of the "surgical insult" of the actual transplant operation itself, this possibility can be reasonably discounted by the findings of a similar significant fall in peripheral IL-4 gene expression in the rejectors with the further administration of potent immunosuppression at the time of acute rejection as discussed in the next paragraph.

At the time of acute rejection, the level of IL-4 gene expression remained the same as the pre-transplant baseline, but immediately following the completion of anti-rejection therapy (the early post-anti-rejection therapy time point), peripheral IL-4 gene expression was again significantly reduced to a level below the pre-transplant baseline, possibly reflecting the potent effect on IL-4 gene expression of the additional immunosuppression received by the rejectors [6.1.3]. However, IL-4 gene expression returned more promptly back to the pre-transplant baseline by 1 week after anti-rejection therapy was completed, and remained at this baseline level of gene expression at the 4 weeks post-anti-rejection therapy time point with the recovery of the allograft from the acute rejection episode.

Thus, we have not been able to demonstrate a difference in IL-4 gene expression at the time of acute rejection in rejectors when compared with the non-rejectors. However, the data suggests that peripheral monitoring of IL-4 gene expression may be correlated with the responses to immunosuppressive therapy and it appears that the changes in peripheral IL-4 gene expression do mirror the changes in the degree of immunosuppression achieved rather than the changes in T cell alloreactivity. Moreover, with the fall in peripheral IL-4 gene expression coinciding with the high level of immunosuppression at the early post-transplant time point, it may be inferred that IL-4 appears to behave more as a proinflammatory cytokine than an immunosuppressive cytokine [Krams et al, 1992; Whitehead et al, 1993].

7.1.3 IL-10

As was demonstrated in the previous chapter, the trend of changes in peripheral T cell IL-10 gene expression appeared to be opposite to that seen for IL-4. It is interesting to note that while peripheral IL-4 gene expression fell significantly at the early post-transplant time point and remained significantly below the pre-transplant baseline level at the 1 week post-transplant time point for both non-rejectors and rejectors, in contrast, peripheral IL-10 gene expression rose significantly above the pre-transplant baseline at the early post-transplant time point and remained significantly above that level at the 1 week post-transplant time point in both groups of patients [6.3.3]. If IL-4 appears to behave more as a proinflammatory cytokine, then IL-10 would appear to behave more as an immunosuppressive cytokine since the significant increase in IL-10 gene expression was in tandem with the high level of immunosuppression present during the first week following renal transplantation.

This finding that IL-10 may be an immunosuppressive cytokine is in contrast to the recent intragraft gene expression studies which associated IL-10 with acute renal allograft rejection [Xu et al, 1995; Strehlau et al, 1997; Suthanthiran, 1997]. The different conclusion drawn on the role of IL-10 may be explained by the differences between our study and those quoted previously, since our finding was based on a

sequential, peripheral T cell gene expression study while those quoted previously were single time-point intragraft gene expression studies.

The most important difference between the non-rejectors' and the rejectors' peripheral T cell IL-10 gene expression profiles was that while the level of IL-10 gene expression remained consistently elevated above the pre-transplant baseline level in the non-rejectors, and indeed increased further at 4 weeks post-transplant [6.1.4], in the rejectors however, by 2 weeks post-transplant [6.3.3] and at the time of acute rejection (the pre-anti-rejection therapy time point) [6.1.4], the level of IL-10 had returned to the level of the pre-transplant baseline. The consistently high level of IL-10 expression seen in our non-rejecting cohort throughout the post-transplant period is supported by the similar findings of high levels of endogenous IL-10 production seen in experimental models of tolerance [Roncarolo, 1995; Roncarolo et al, 1996]. One could surmise that there was a relative decrease in the efficiency of immunosuppression at the cytokine level in the rejectors (even though both groups of patients were equally immunosuppressed pharmacologically based on their cyclosporin A profiles as shown in figures 5.5.9 and 5.5.10 in chapter 5) to account for this difference in IL-10 gene expression profiles between the 2 groups of patients. It is therefore not inconceivable that this relative decrease in peripheral IL-10 gene expression may be the signal indicating the impending onset of an acute allograft rejection process in the patients who subsequently go on to experience acute rejection.

One weakness of the study was the limited number of data available for analysis at 2 weeks post-transplant in the rejectors, which may prompt critics to dismiss the return to the pre-transplant baseline level of IL-10 gene expression seen at that time point. However, a similar relative decrease in peripheral T cell IL-10 gene expression (when compared with the non-rejectors' peripheral IL-10 gene expression profile) to pre-transplant baseline level at the time of acute rejection seen in the rejectors (based on 14 out of 15 rejectors' data at the pre-anti-rejection therapy time point available for analysis) would support the contention that the relative decrease in peripheral IL-10 gene expression at 2 weeks post-transplant was indeed a true reflection of the

changes in T cell alloreactivity prior to, or during, an acute rejection process. The subsequent significant increase in IL-10 gene expression above the pre-transplant baseline at the completion of anti-rejection therapy (the early post-anti-rejection therapy time point) points to the significant effect that the additional immunosuppressive therapy had on IL-10 gene expression. Overall, these results suggest that IL-10 may be another cytokine which is not only sensitive to changes in the level of immunosuppression, albeit an opposite one to IL-4, but that it may be a useful marker in distinguishing between the two groups of patients when the level of expression of this cytokine is monitored sequentially. However, based on current evidence, it cannot be said that the pattern of IL-10 gene expression could be a predictive marker of acute rejection since the overall number of rejectors in our study cohort was relatively small compared with the number of non-rejectors.

Finally, it is interesting to note that with the successful treatment of acute rejection, the profile of IL-10 gene expression in the rejectors did not return to the profile seen in the non-rejectors (i.e., a level of IL-10 consistently above the pre-transplant baseline) as one would expect, but instead the level of IL-10 settled back to the pre-transplant baseline level at 1 week and 4 weeks following completion of anti-rejection therapy [6.1.4]. It may be that other factors influencing the cytokine balance during the acute rejection episode, like the reciprocal negative feedback mechanisms between the Th1 and Th2 cytokines described in chapter 1, which may continue to be dominant and therefore be influencing the IL-10 gene expression profile, even though the renal allografts appear to be recovering both clinically and biochemically from the acute rejection episode. As our study did not monitor the peripheral T cell gene expression profiles beyond these time points, we do not know whether IL-10 gene expression would ultimately return to the pattern seen in the non-rejectors.

7.1.4 IL-5

The association of IL-5 with acute allograft rejection has been demonstrated by intragraft gene expression studies in clinical liver [Martinez et al, 1992; Martinez et

al, 1993a; Martinez et al, 1993b], renal [Krams et al, 1992] and lung transplantation [Whitehead et al, 1993], and in murine cardiac transplantation [Chan et al, 1995]. IL-5 products in bile and serum were also found to be elevated in rejecting liver allograft recipients [Lang et al, 1995].

We have found that IL-5 is another cytokine whose sequential gene expression profiles in peripheral T cells have demonstrated substantive differences between non-rejectors and rejectors. While the overall pattern in peripheral IL-5 gene expression in non-rejectors remained significantly and consistently below the pre-transplant baseline level at all post-transplant time points [6.1.5], this was not so in the rejectors. Although the early post-transplant level of IL-5 gene expression of peripheral T cells in the rejectors was below the pre-transplant baseline as in the non-rejectors, the level of peripheral IL-5 gene expression had returned to the pre-transplant baseline at the 1 week and 2 weeks post-transplant time points [6.3.4], in contrast to the profile seen in the non-rejectors at these time points. This relative rise in IL-5 gene expression in the rejectors may have provided the requisite trigger in initiating or facilitating the occurrence of the subsequent acute rejection episodes experienced by this group of patients, since IL-5 has been reported to be a proinflammatory cytokine by its effects on eosinophil activation and function [reviewed in 1.5.5].

Moreover, whilst the level of peripheral IL-5 gene expression in the rejectors was at the pre-transplant baseline level at the time of rejection (the pre-anti-rejection therapy time point), which was a relatively higher level of gene expression when compared with the general post-transplant level of gene expression seen in the non-rejectors, the beneficial effect of anti-rejection therapy was seen by the level of peripheral IL-5 gene expression falling significantly back below the pre-transplant baseline again at all time points following the completion of anti-rejection therapy [6.1.5], a pattern similar to that seen in the non-rejectors.

These differences in the profiles of peripheral IL-5 gene expression between the two groups suggest that IL-5 may be important as a cytokine marker in differentiating those patients who subsequently experience an acute allograft rejection episode.

7.1.5 IL-13

Although IL-13 has a lot in common with IL-4 in terms of its spectrum of biological activities and indeed may even share a common receptor or receptor component with IL-4 [as reviewed in 1.5.6], the gene expression profiles for IL-13 in peripheral T cells did not mirror the profiles seen for IL-4, and indeed showed fewer changes between time points than for IL-4.

Like the other Th2 cytokines, IL-13 has both proinflammatory and immunosuppressive properties [reviewed in 1.5.6]. However, the role of IL-13 in transplantation immunology remains unknown and has not been systematically investigated in any study published to date.

Unlike those patients who did not experience acute allograft rejection, a trend towards increased peripheral IL-13 gene expression following renal transplantation was seen in the rejectors, with the level at the 2 weeks post-transplant time point and at the time of acute rejection (the pre-anti-rejection therapy time point) being significantly higher than the pre-transplant baseline [6.1.6 and 6.3.5]. This suggests an upregulation in IL-13 gene expression with impending or during acute rejection, since in contrast, the non-rejectors' gene expression profile for IL-13 was rather "flat" with no significant changes at all time points following renal transplantation [6.1.6].

The potent effect of anti-rejection therapy on peripheral IL-13 gene expression was evidenced by the significant decrease in IL-13 gene expression back to the pre-transplant baseline level at the completion of anti-rejection therapy (the early post-anti-rejection therapy time point), and this level of IL-13 gene expression was maintained at the pre-transplant baseline level at 1 week and 4 weeks following the completion of anti-rejection therapy [6.1.6]. Interestingly, unlike for IL-4, there was no decrease in IL-13 gene expression at the early post-transplant time point in both groups of patients with the loading dose of immunosuppressive drugs.

Thus, IL-13 appears to be quite different in its sequential profile from IL-4, and like IL-5, may be an important cytokine in distinguishing between the rejectors and the non-rejectors.

7.1.6 GrB

The demonstration of functionally active CTLs accumulating within rejecting allografts in clinical renal transplantation [Strom et al, 1975] and experimental cardiac transplantation [Strom et al, 1977] in the early days, and the subsequent findings of intragraft GrB gene transcripts, a marker of CTL activation, in acutely rejecting allografts in clinical renal transplantation [Lipman et al, 1992; Lipman et al, 1994; Sharma et al, 1996; Strehlau et al, 1996; Strehlau et al, 1997; Suthanthiran, 1997] have raised hopes that GrB may be a marker of acute allograft rejection.

In our study, the only significant difference in the peripheral T cell GrB gene expression profiles between non-rejectors and rejectors was the early fall in GrB expression seen in the rejectors but not in the non-rejectors [6.1.7]. As a similar parallel early fall in the two proinflammatory Th1 cytokines studied in the project (IL-2 and IFN-γ) was also noted only in the rejectors as discussed in sub-section 7.1.1, one could speculate that GrB, being a marker of CTL activation, may be an important marker suggesting an early sequestration of activated CTLs into the allografts in patients who subsequently experience acute allograft rejection.

However, it is difficult to explain why the level of peripheral GrB gene expression had decreased to a level significantly below the pre-transplant baseline in the non-rejectors at the 1 week and 2 weeks post-transplant time points [6.1.7], since it is not possible to use the same hypothesis of sequestration of activated CTLs into the allografts in the rejectors at the early post-transplant and 1 week post-transplant time points [6.3.6]. These seemingly random changes in peripheral GrB gene expression in both groups of patients demonstrated by our data suggest that GrB may not be a useful marker in the immunological monitoring of patients following renal transplantation.

7.1.7 FasL

Like GrB, FasL is another marker of CTL activation whose intragraft [Sharma et al, 1996; Strehlau et al, 1996; Strehlau et al, 1997] and peripheral blood leucocyte [Vasconcellos et al, 1998] gene expression had been correlated with acute allograft rejection in clinical renal transplantation. However, recent studies have produced more conflicting findings, with gene expression studies in experimental renal [Wang et al, 1997] and cardiac [Seino et al, 1996; Josien et al, 1998] transplantation supporting a significant contribution of FasL to acute allograft rejection, while in a murine non-vascularized heterotopic cardiac allograft model, Fas/FasL-mediated cytotoxicity was shown not to be required for murine cardiac allograft rejection [Djamali and Odorico, 1998], and in a murine skin allograft transplant model, it was shown that while FasL gene expression correlated closely with rejection, FasL was not required for allograft rejection [Borson et al, 1999]. A recent study in clinical liver transplants [Tannapfel et al, 1999] had found that increased apoptosis, Fas, and FasL expression were, taken by themselves, not useful as indicators of acute rejection.

Overall, the peripheral FasL gene expression profile in the rejectors prior to the acute rejection episodes was very similar to the FasL profile seen in the non-rejectors over the same protocol sampling time points [6.3.7]. This similarity, coupled with the surprisingly "flat" FasL gene expression profile in the rejectors at the time of acute rejection and following the completion of anti-rejection therapy [6.1.8], suggests that the only difference seen in FasL gene expression level between the 2 groups at the 2 weeks post-transplant time point may not be of any value in differentiating the rejectors from the non-rejectors [6.3.7].

Based on the results of our study, we conclude that FasL, like GrB, may not be a useful marker in the immunological monitoring of patients following renal transplantation.

7.2 Relationships between the changes in the level of the individual cytokine/CTL activation marker gene expression at each sampling time point

In the previous section, we discussed the sequential changes in peripheral T cell gene expression and the differences in the individual cytokine/CTL activation marker gene expression profiles between the non-rejectors and the rejectors following renal transplantation. In this section, we summarise the individual changes in gene expression of each cytokine/CTL activation marker at each sampling time point in order to examine the relationships between individual cytokines/CTL activation markers. For convenience, these relationships will be discussed in 3 separate sub-sections, namely, those seen in the non-rejectors, those seen in the rejectors prior to the acute rejection episodes, and finally, those seen at the time of acute rejection and in the time period following completion of anti-rejection therapy.

7.2.1 Relationships between the cytokines/CTL activation markers in the non-rejectors

In the absence of any additional immunological stimuli as in the processes leading up to acute allograft rejection, the changes in the level of cytokines/CTL activation markers gene expression at each sampling time seen in the non-rejectors should reflect the outcome of the circulating T cells' exposure to the alloantigens of the transplanted allograft and its modulation by the immunosuppression received.

The levels of IL-2, IFN-γ and IL-13 expression remaining at their respective pre-transplant baselines at all time points in the post-transplant period in the non-rejectors suggest that the presence of the allograft and the immunosuppressive agents did not influenced these 3 cytokines to any significant degree. It is unlikely that the lack of changes in these cytokines were the result of a failure to detect the changes since significant changes in all 3 cytokines were demonstrated in the rejectors.

IL-4 and IL-5 gene expression appeared to move in tandem initially with a significant fall at the early post-transplant time point, while IL-10 expression was in the opposite direction, increasing significantly from the pre-transplant baseline. The initial parallelled decreased expression of IL-4 and IL-5 quickly diverged, with the level of IL-4 expression reversing in trend from 1 week post-transplant onwards by gradually and significantly increasing in expression back to the pre-transplant baseline level, but the levels of IL-5 and IL-10 expression remained at their respective levels at 1 and 2 weeks post-transplant time points. While IL-5 expression remained significantly below its pre-transplant baseline throughout the entire post-transplant period, the level of IL-10 expression continued to be significantly above its pre-transplant baseline throughout, and indeed increased significantly further at 4 weeks post-transplant.

The starkly different changes in the level of peripheral T cell IL-4, IL-5 and IL-10 gene expression in the non-rejectors demonstrate the varying effect of immunosuppressive agents on gene expression levels of these cytokines. While the "suppression" of IL-4 gene expression was transient, the same effect on IL-5 was prolonged throughout the post-transplant period. In contrast, the effect on IL-10 gene expression was the opposite, with ever increasing level of expression, possibly reflecting the adequacy in the degree immunosuppression present in the non-rejectors and the resulting absence of acute allograft rejection. Our results for IL-5 and IL-10 are in keeping with the known role of IL-10 in inhibiting IL-5 synthesis by T cells [Pretolani and Goldman, 1997].

As for the 2 CTL activation markers, both their non-rejectors' profiles were very similar by being relatively "flat" [6.1.7 and 6.1.8], with no significant sequential changes from pre-transplant baseline to 2 weeks post-transplant followed by a significant increase in expression at 4 weeks post-transplant from the previous time point. However, while the level of GrB expression were significantly below its pre-transplant level at 1 and 2 weeks post-transplant, for FasL, only the 1 week post-transplant level was significantly below its pre-transplant level. Nevertheless, both GrB and FasL expression at 4 weeks post-transplant were back at their

respective pre-transplant baseline levels. It would appear from these findings that neither the the allograft or the immunosuppressive agents had any significant impact on the 2 CTL activation markers gene expression.

7.2.2 Relationships between the cytokines/CTL activation markers in the rejectors prior to acute rejection

In this subgroup of results from the rejectors, the presence of the allograft coupled with the ultimate failure of the immunosuppressive agents to prevent acute allograft rejection clearly have significant and diverse impacts on the levels of gene expression of the cytokines and CTL activation markers at the various sampling time points up to 2 weeks post-transplant (prior to the acute rejection episode).

In the rejectors over this period, the levels of IL-2, IFN-γ, IL-4 and IL-5 expression all decreased significantly at the early post-transplant time point, while IL-10 increased significantly, but IL-13 remained at its pre-transplant baseline level. At the 1 week post-transplant time point, while IL-2, IFN-γ and IL-5 remained unchanged at below pre-transplant baseline levels, both IL-4 and IL-10 reversed their previous trends by respectively increasing and decreasing significantly (IL-4 remaining significantly below its pre-transplant baseline and IL-10 significantly above its pre-transplant baseline). IL-13 also increased significantly, but it nevertheless remained at its pre-transplant baseline level at the 1 week post-transplant time point. By 2 weeks post-transplant, all cytokines had drifted back to their respective pre-transplant baselines except for IL-13, which was significantly above its pre-transplant baseline.

It is difficult to explain why the 2 proinflammatory Th1 cytokines (IL-2 and IFN-γ) was "suppressed" below their respective pre-transplant baseline in the rejectors as one would have expected the reverse. The possibility of sequestration of these Th1 cells into the allograft to account for these changes in IL-2 and IFN-γ was discussed section 7.1.1.

With the exception of IL-4, the changes in the other cytokines could possibly be explained by the effect of immunological processes leading up to the impending acute rejection. By returning to their pre-transplant baselines, IL-5 expression was relatively higher, while IL-10 expression was relatively lower than that seen at the same time points in the non-rejectors. This reversal in the levels of IL-5 and IL-10 could be explained by a reduction in the inhibitory effect of IL-10 (with reduced expression) resulting in an increase in IL-5 expression [Pretolani and Goldman, 1997]. The level of IL-13 expression, on the other hand, had clearly increased significantly above its pre-transplant baseline in contrast to that seen in non-rejectors again. The combination of these increases in IL-5 and IL-13 gene expression may therefore be significant markers heralding the imminent acute rejection process. The common influence of both cytokines on eosinophil function [reviewed in sections 1.5.5 and 1.5.6] could suggest a role for allergic inflammation involving eosinophils as part of the pathological process of acute allograft rejection, as was suggested by Martinez and her co-workers [Martinez et al, 1993a] based on their work on intragraft expression of IL-5 in liver transplantation.

IL-4 was the only cytokine in the rejectors to behave in the same manner as in the non-rejectors at all time points from pre-transplant to 2 weeks post-transplant. Therefore, it would appear that although peripheral T cell IL-4 gene expression do vary significantly at different time points following transplantation, the impending immunological processs leading to the acute rejection did not affect the level of IL-4 expression in any way. In the midst of conflicting findings on the role of IL-4 in acute allograft rejection, our results for IL-4 have added further support to those studies which did not find an association of IL-4 with acute allograft rejection.

For the CTL activation markers, GrB decreased significantly at the early post-transplant time point while FasL remained at its pre-transplant baseline. At 1 week and 2 weeks post-transplant time points, although there were no further significant sequential changes for both CTL activation markers, GrB initially remained significantly below its pre-transplant baseline before drifting back to its baseline, but FasL had drifted significantly below its pre-transplant baseline at both

time points. The significance of the early fall in GrB but not in FasL is unclear. This, coupled with the apparently reversed levels of expression of both CTL activation markers for no clear reason would suggest that as in the non-rejectors, the changes in the level of the CTL activation markers gene expression in the rejectors are not helpful as markers of the impending acute rejection process.

7.2.3 Relationships between the cytokines/CTL activation markers in the rejectors during acute rejection and following anti-rejection therapy

The gene expression levels of the cytokines or CTL activation markers at the time of acute rejection (the pre-anti-rejection therapy time point in our study) can reveal important relationships between the individual cytokines or CTL activation markers and the acute rejection process, although we recognised that the timing of these samples in relation to the timing and stage of the acute rejection process may be variable between the patients. Nevertheless, the changes in the levels of cytokines/CTL activation markers gene expression at the time of acute rejection, and in addition, the changes seen in the time period following anti-rejection therapy has the potential to provide important information about the role of the individual cytokine/CTL activation marker in acute allograft rejection.

At the time of acute rejection, the levels of IFN-γ, IL-4, IL-5 and IL-10 gene expression were not significantly different from their respective pre-transplant baselines. However, the levels of IL-2 and IL-13 were significantly below and above their pre-transplant baselines respectively. Clearly the ongoing acute rejection process had a significant impact on the level of peripheral T cell IL-5, IL-10 and IL-13 gene expression, but did not affect IFN-γ and IL-4 expression. The similar increase in IL-5 and IL-13 expression (in relative or absolute terms respectively), coupled with a reduced IL-10 expression, as seen in the time points preceding acute rejection described previously [7.2.2], further reinforces the importance of IL-5 and IL-13 as markers of the acute rejection process. However, it is difficult again to explain the reduction in IL-2 expression at this time point, except for the possibility of further sequestion of IL-2 producing Th1 cells into the allograft to account for this.

The additional immunosuppression given to the rejectors to treat the acute rejection process clearly had a potent effect on cytokine gene expression, as seen by the marked changes in the levels of most of the cytokines studied following the completion of the anti-rejection therapy (the early post-anti-rejection therapy time point). IFN-γ, IL-4 and IL-5 decreased significantly below their pre-transplant baselines, while IL-10 increased significantly above its pre-transplant baseline and IL-13 decreased significantly back to its pre-transplant baseline. This downregulation of IFN-γ, IL-4, IL-5 and IL-13 gene expression with the additional immunosuppression mirrors the downregulatory effect of corticosteroids on the gene expression and protein secretion of these cytokines by peripheral blood mononuclear cells [Braun et al, 1997]. IL-2 was the only cytokine to remain unchanged by persisting below its pre-transplant baseline. The reversal in the expression of IL-5, IL-10 and IL-13 to that seen in the non-rejectors from their respective levels prior to commencing anti-rejection therapy suggests that the expression of these 3 cytokines do mirror closely the changes in the immunological status within the allograft and the dampening down of the acute rejection process. Moreover, the changes in IL-5 and IL-13 expression before and after anti-rejection therapy mirror the findings of blood eosinophilia following renal transplantation at the time of acute cellular rejection episodes and its down-regulation following steroid pulse therapy by Lautenschlager et al [1985], further supporting the role of eosinophils in acute allograft rejection.

The decrease in IL-4 seen at this time point is reminiscent of the changes seen in both groups of patients at the early post transplant time point, and further signify the sensitivity of this cytokine to immunosuppressive agents. Although the additional immunosuppression had affected IFN-γ but not IL-2 expression, the overall variable changes in both cytokines make it difficult to interprete the changes in these cytokines at this particular time point.

However, the initial potent effect of the additional immunosuppression on the levels of cytokine gene expression seen at the early post-anti-rejection therapy time point had variable lasting effects on the different cytokines by 1 week and 4 weeks

following completion of anti-rejection therapy. All the cytokines appeared to "drift" back to their respective pre-transplant baseline level of expression, except for IL-5, which persisted significantly below its pre-transplant baseline at both time points. While the successful reversal of the acute rejection process clearly had return IL-5 and IL-13 gene expression to the levels seen in the non-rejectors, this was not so for IL-10. Nevertheless, despite IL-10 expression not being elevated at these time points, the level of IL-5 remained "suppressed", indicating that the relationship between the expression of IL-10 and IL-5 may not be a direct one. As for IL-4 returning back to its pre-transplant baseline again, this could be a marker that the level of immunosuppression had returned to the "steady-state", as in the non-rejectors by 2 weeks post-transplant. There is nothing particularly noteworthy for the 2 Th1 cytokines.

As for GrB and FasL, neither the acute rejection process itself nor the anti-rejection therapy had any effect on the level of gene expression of either CTL activation markers with both GrB and FasL remaining at their respective pre-transplant baseline levels at all time points before and after anti-rejection therapy.

7.3 Sequential changes in cytokine ratios

This section discusses the profiles of sequential changes in cytokine ratios as set out in section 6.2 of the previous chapter. Overall, there were not as many, nor as dramatic, changes seen in the profiles of cytokine ratios compared with the individual cytokine profiles. The cytokine ratio that had the most interesting sequential profiles for both rejectors and non-rejectors was the IL-10/IL-4 ratio, and this is discussed in the first sub-section. Note that only the ratios of Th2 to Th1 cytokines and between the individual Th2 and Th1 cytokines from the first phase of the study (i.e. IL-2, IFN-γ, IL-4 and IL-10) were analysed.

7.3.1 IL-10/IL-4 Ratio

Several important differences in the pattern of changes in IL-10/IL-4 ratio were seen between the non-rejectors and rejectors and these are highlighted in this sub-section. Indeed the changes in IL-10/IL-4 ratio were more prominent in terms of the magnitude of the changes than when either cytokine was considered separately, and this was caused principally by the opposing pattern of changes between IL-10 and IL-4 gene expression at each time point, thus producing the resulting IL-10/IL-4 profiles in rejectors and non-rejectors that were basically the accentuated profiles of peripheral IL-10 gene expression alone.

The sharp rise in IL-10/IL-4 ratio in both groups of patients at the early post-transplant time point [6.2.1], as for the parallel changes seen in both groups of patients at this time point for the 2 cytokines alone, could again be interpreted as reflecting the loading dose effect of the immunosuppressive drugs. It should be noted that the levels of IL-10/IL-4 ratio in non-rejectors were consistently above the pre-transplant baseline at all post-transplant time points [6.2.1], just as the non-rejectors' profile for peripheral IL-10 gene expression alone had the same pattern [6.1.4]. In contrast, in the rejectors, the IL-10/IL-4 ratio returned to the pre-transplant baseline level at 2 weeks post-transplant and at the time of acute rejection (the pre-anti-rejection therapy time point) [6.3.8 and 6.2.1], mirroring the rejectors' pattern of changes in peripheral IL-10 gene expression alone [6.3.3 and 6.1.4]. This suggests a balance between these two Th2 cytokines such that the IL-10/IL-4 ratio remaining elevated above the pre-transplant baseline level in the time period following renal transplantation may be an important factor in preventing the development of acute allograft rejection.

Moreover, the IL-10/IL-4 ratio rose significantly above the pre-transplant baseline following the completion of anti-rejection therapy (the early post-anti-rejection therapy time point), mirroring the significant rise in IL-10/IL-4 ratio seen at the early post-transplant time point in both groups of patients [6.2.1]. Therefore, this rise in IL-10/IL-4 may be a reflection of the effect of additional immunosuppression in

altering the cytokine balance in peripheral T cells back towards that seen in the non-rejectors' profile. However, it was clear from the results set out in sub-section 6.2.1 that despite this initial rise in IL-10/IL-4 ratio following the completion of anti-rejection therapy, the IL-10/IL-4 ratio in the rejectors did not return to a level above the pre-transplant baseline (as seen in the non-rejectors' profile) even though the acute rejection episode had been successfully treated. This pattern is again the same as that seen for the rejectors' peripheral IL-10 gene expression profile [6.1.4].

The data from the IL-10/IL-4 ratio in our study suggest that the alteration in cytokine balance caused by the acute rejection episode may persist for several weeks following the cessation of the trigger that caused the initial alteration in cytokine balance in the first place. Since no samples were taken beyond 4 weeks following the completion of anti-rejection therapy, we do not know whether this level of IL-10/IL-4 ratio would continue to be maintained at the pre-transplant baseline level in the long term, or that the IL-10/IL-4 ratio will slowly return back to the non-rejectors' profile.

7.3.2 IL-4/IL-2 Ratio

There were only two main differences in the IL-4/IL-2 ratio profiles of rejectors and non-rejectors. While there was a significant fall in IL-4/IL-2 ratio below the pre-transplant baseline at the early post-transplant time point in the non-rejectors, the IL-4/IL-2 ratio remained at the pre-transplant baseline level in the rejectors [6.2.2]. This difference is principally due to the significant fall in IL-2 gene expression seen in the rejectors but not in the non-rejectors at the early post-transplant time point as described previously [6.1.1].

The other finding of note was the increase in IL-4/IL-2 ratio at the time of acute rejection (the pre-anti-rejection therapy time point) above the pre-transplant baseline, and this rise in IL-4/IL-2 ratio appears to persist long after the completion of successful anti-rejection therapy as seen by the IL-4/IL-2 ratio remaining significantly above the pre-transplant baseline at the 1 week and 4 weeks post-anti-rejection therapy time points [6.2.2]. These changes were basically the same as that seen for

peripheral IL-4 gene expression alone but with the changes "ironed out" and elevated above the pre-transplant baseline due to the low level of peripheral IL-2 gene expression. As a result, the acute rejection process appears to have elevated the IL-4/IL-2 ratio but treatment of the acute rejection did not seem to reverse this trend.

Despite these clear differences seen between the two IL-4/IL-2 ratio profiles for each patient group, it is difficult to explain the reasons for them since the changes do not follow the clinical course as clearly as seen for the individual cytokines. It may be that these changes in IL-4/IL-2 ratio were simply artefacts of this method of analysis, or it implies that taking Th2 to Th1 cytokine ratio as a means of elucidating the Th2/Th1 balance did not contribute in any way to the immunological monitoring of our patients following renal transplantation.

7.3.3 IL-4/IFN-γ Ratio

In contrast to IL-4/IL-2 ratio profiles, there did not seem to be any difference between the rejectors and non-rejectors in their IL-4/IFN-γ ratio profiles during the first week following renal transplantation. However, at the 2 weeks post-transplant time point, IL-4/IFN-γ ratio was significantly higher than the pre-transplant baseline in the rejectors but not in the non-rejectors [6.3.10]. As this was the only significant difference between the 2 groups of patients, it should be interpreted cautiously, especially since the number of samples available for analysis at this time point was small in the rejectors. Moreover, our caution was perhaps justified seeing that the IL-4/IFN-γ ratio was not significantly different from the pre-transplant baseline at the time of acute rejection and all the subsequent time points following completion of anti-rejection therapy [6.2.3].

Therefore, as with the situation for IL-4/IL-2 ratio, the changes in IL-4/IFN-γ do not appear to follow the clinical course of our study cohort following renal transplantation. These data enhanced our conclusion that taking Th2 to Th1 cytokine ratio as a means of elucidating the Th2/Th1 balance may not be a useful method to monitor our patients immunologically following renal transplantation.

7.3.4 IL-10/IL-2 & IL-10/IFN-γ Ratios

Overall, there were no major differences in the sequential profiles of both IL-10/IL-2 and IL-10/IFN-γ ratios that would distinguish the rejectors (over the protocol time points prior acute rejection) from the non-rejectors over the same time points [6.3.11]. The 2 ratios were similarly unremarkable at the time of rejection (the pre-anti-rejection therapy time point) and the time points following anti-rejection therapy [6.2.4 and 6.2.5], and the changes do not appear to correlate well with the clinical course of the patients in the light of the data obtained for peripheral IL-10 gene expression alone.

It is likely that these aberrations seen in the data for both IL-10/IL-2 and IL-10/IFN-γ ratios were artefactual, and as concluded in the discussions on the data for the other 2 Th2/Th1 analyses [7.3.3 and 7.3.4], we believe that the determination of Th2/Th1 balance by taking Th2 to Th1 cytokine ratio using these cytokines may not be of any value as a means of immunological monitoring in patients renal transplantation. This is in stark contrast to the determination of Th2 balance [7.3.1] which we have found to be extremely useful, since the sequential changes noted for the individual Th2 cytokines were accentuated when their ratios were analysed.

7.3.5 IFN-γ/IL-2 Ratio

This ratio was done mainly for completeness of the analysis to see if taking the ratio of the two Th1 cytokines would yield any interesting findings. Not surprisingly, there were no major differences in the IFN-γ/IL-2 ratio profiles between the two groups of patients, especially prior to the acute rejection episode. There appeared to be a persistent increase in IFN-γ/IL-2 ratio with the during the acute rejection episode and following its successful reversal, although the reasons for this are unclear. Overall, the balance between these two Th1 cytokines is probably not a useful marker to distinguish between the two groups of patients.

7.3.6 Concluding remarks about cytokine ratios

From the above discourse concerning the relevance or otherwise of cytokine ratios in the immunological monitoring of patients following renal transplantation, it was clear that the only cytokine ratio that reflects the progressive stages in the clinical course of our study cohort most closely was the ratio between the 2 Th2 cytokines, IL-10 to IL-4. The other cytokine ratios (i.e., the 4 ratios of Th2 to Th1 cytokines and the ratio between the 2 Th1 cytokines) did not appear to correlate much with the clinical course of the patients. The reason for this difference between IL-10/IL-4 ratio and the other cytokine ratios may be that only IL-10 and IL-4 appeared to have clearly opposite patterns of gene expression profiles when compared with each other, while the other comparisons between the cytokines appeared to be more random.

7.4 Comparison between non-rejectors and rejectors at all protocol time points prior to acute rejection

Overall, the statistical (Mann-Whitney U test) comparisons of the data at all protocol time points for all cytokines/CTL activation markers or cytokine ratios between non-rejectors and rejectors did not show any significant differences except for the following: IL-2 at all 3 post-transplant time points, IL-13 at the 2 weeks post-transplant time point and IL-4/IFN-γ at 1 week and 2 weeks post-transplant time points. This suggests that the general level of peripheral T cell gene expression in the 2 groups of patients were essentially similar at all time points prior to the acute rejection episode and therefore the 2 groups of patients were comparable.

The difference in IL-2 gene expression at all 3 post-transplant time points between the two groups is difficult to interprete clearly since the overall level of IL-2 gene expression was generally low in comparison with the other cytokines studied. Moreover, although the IFN-γ gene expression profile showed similar changes to IL-2 as discussed previously [7.1.1], no significant differences in IFN-γ gene expression profile between the two groups of patients can be demonstrated by the

Mann-Whitney analysis. This suggests that the difference in IL-2 gene expression between the 2 groups of patients was unique to this cytokine only, since the other cytokines (except for IL-13 at one time point) and CTL activation markers studied in the project did not show any significant differences. The reason why this should be the case is unclear.

As for the significant difference detectable for IL-13 at the 2 weeks post-transplant time point, it is difficult to explain why the level of peripheral IL-13 gene expression in rejectors should be significantly higher than in the non-rejectors at this time point. Although it is possible that of all the cytokines and CTL activation markers studied in the project, the magnitude of change in the level of IL-13 gene expression as compared with its pre-transplant baseline could potentially be an important factor in identifying patients with impending acute rejection, we would be cautious in interpreting the Mann-Whitney analysis in this way since the number of data available for analysis at the 2 weeks post-transplant time point for the rejector group was small as a sizeable number of patients in the rejectors' group had experienced acute rejection by the 2 weeks post-transplant time point.

The two significant findings on Mann-Whitney analysis for IL-4/IFN-γ ratio at the 1 week and 2 weeks post-transplant time points (with the IL-4/IFN-γ ratio being higher in the rejectors than in the non-rejectors) are also difficult to interprete. As the sequential IL-4/IFN-γ ratio profiles for both rejectors and non-rejectors over the same protocol time points (except for the 2 weeks post-transplant time point discussed previously in 7.3.3) were very similar, these findings are unlikely to have any clinical relevance, particularly as the sequential profile for IL-4/IFN-γ ratio at the time of acute rejection and all the time points following completion of anti-rejection therapy did not reveal any significant changes at all in this cytokine ratio.

7.4.1 Conclusions

The preceding discussion pointing out the general lack of statistical difference in the Mann-Whitney analysis between the non-rejectors and rejectors at all protocol time

points prior to acute rejection has served to highlight the importance of the sequential analysis in demonstrating important differences in some of the cytokine gene expression profiles between non-rejectors and rejectors reported in section 7.1 which was clearly not possible to detect in the analysis directly comparing gene expression levels of the two cohorts at the same sampling time point. This could perhaps also explain the reason why conflicting findings about the significance of individual cytokines in acute rejection is so common amongst the numerous single time point cytokine gene expression studies.

Chapter 8: GENERAL DISCUSSION AND CONCLUSIONS

This project has achieved the objectives set out at its inception, namely, the successful development of an efficient method of isolating peripheral blood T cells for molecular analysis, the establishment of a RT-PCR ELISA methodology as a semi-quantitative assay which is reliable, accurate and reproducible, and the application of this methodology to study sequential cytokine and CTL activation marker gene expression profiles of peripheral blood T cells. The results from this study have revealed fresh insights into the patterns of peripheral T cell gene expression in both patients who did not experience acute allograft rejection episodes and those who did.

The preliminary cell separation experiments detailed in chapter 3 showed that T cells can be easily and reliably obtained from peripheral blood using a combination of well established cell separation techniques described in that chapter, and by modifying these cell separation techniques, the cell separation component of the overall project methodology has facilitated the isolation of peripheral blood T cells from our study patients in the shortest time possible so that the risk of inadvertent stimulation of the peripheral blood T cells thus isolated during the course of the cell separation has been kept to a minimum. However, we did not examine the isolated T cells for the expression of activation markers such as IL-2 receptor. The cell separation methodology used in the project has also been successful even in patients with low lymphocyte counts, as some of our patients were, especially following the completion of biological agent anti-rejection therapy.

As may be surmised from the number of preliminary experiments performed on the molecular aspects of the project as described in chapter 4, it was important to ensure that this component of the project was thoroughly tested, fine-tuned and validated prior to analysing the study samples using this methodology. The conditions for the PCR component of the RT-PCR ELISA methodology were optimised and the overall

RT-PCR ELISA methodology was checked for its reliability in providing an assay of the sequential changes in the level of cytokines and CTL activation markers gene expression from peripheral blood T cells that is truly semiquantitative. This was done by demonstrating that an increasing amount of starting cDNA for the RT-PCR ELISA assay corresponded closely with an increasing amount of PCR products detected and vice versa. Confidence in the reliability of the RT-PCR ELISA methodology was further strengthened by the demonstration that the results obtained using this method were easily reproducible, in terms of obtaining the same pattern of sequential changes when the RT-PCR ELISA methodology was applied on the same set of samples at different times, and also when a set of samples were assayed at different dilutions. These preliminary experiments also demonstrated that the RT-PCR ELISA methodology is a very sensitive assay capable of detecting PCR products from highly diluted cDNA samples. Ideally, we should have performed studies to determine the half-life of the mRNA for all the cytokines and CTL activation markers studied in the project.

All the preliminary molecular experiments convinced us that the RT-PCR ELISA methodology developed during the early phase of the project was sufficiently reliable to be used as a semiquantitative assay to enable us to study sequential changes in the level of cytokines and CTL activation markers gene expression from peripheral blood T cells of our study patients. Despite being a relatively complex and lengthy form of assay for the analyses of a considerable number of samples for multiple markers, it proved technically feasible both in terms of overall logistics and cost.

The statistical analyses applied on the data obtained from the project, both clinical and molecular, had been simple and uncomplicated.

An overview of the analysis of the RT-PCR ELISA results from the study patients has confirmed the importance of performing sequential sampling from the same patient at different time points following transplantation, as opposed to the practice of sampling at a single time point (usually at the time of suspected acute rejection) which is so prevalent in the cytokine literature. Our results have demonstrated that an

apparently insignificant level of cytokine or CTL activation marker gene expression at any time point following renal transplantation on its own may actually be clearly significant when analysed in the light of the level of gene expression at other sampling time points in a sequential study. Moreover, it is clear from the analysis of our results that comparing the level of gene expression of one cytokine or CTL activation marker at all post-transplant time points with its corresponding pre-transplant baseline level of gene expression generally yielded more useful information than comparisons between sequential levels of gene expression. We found that significant changes from the pre-transplant baselines may be present without any significant changes between the sequential time points, although sequential changes were important for some of the cytokines studied.

Overall, the profiles of changes obtained from the RT-PCR ELISA data have demonstrated that peripheral monitoring of the changes in T cell gene expression can provide useful information to distinguish the rejectors from the non-rejectors.

Of the cytokines and CTL activation markers studied in the project, the two Th1 cytokines, IL-2 and IFN-γ, showed unexpected differences in their sequential profiles between rejectors and non-rejectors. While an increase in the level of gene expression following transplantation or with the onset of acute rejection would theoretically be the expected behaviour of proinflammatory cytokines like IL-2 and IFN-γ, we found that the peripheral gene expression of both cytokines in our study decreased significantly instead. Although we had speculated that these decreased levels of IL-2 and IFN-γ gene expression in the rejectors at the early post-transplant time point and during the acute rejection period could be explained by a sequestration of these Th1 cells into the renal allografts or other immune compartments in the patients, thus providing or facilitating the cytokine trigger for the subsequent acute rejection episodes, as yet there are no published studies in the literature demonstrating that this phenomenon occurs in clinical transplantation. Nevertheless, this concept of the sequestration of Th1 cells into the allograft and/or other sites of immune activation like the spleen is plausible since the migration of lymphocytes in rat cardiac transplant models has been shown to be influenced dramatically by the

immunological status of the recipients of vascularized organ allografts [Kupiec-Weglinski et al, 1982]. Moreover, the sequestration of activated CTLs into the allografts has also been suggested as a possible explanation for the finding of a considerably higher frequency of donor-specific CTLs in the graft cell population when compared with the peripheral blood lymphocytes in clinical cardiac transplantation [Suitters et al, 1990].

The only group of workers who have found a similar fall in peripheral IL-2 gene expression (albeit induced expression) at the time of acute rejection episodes in both human and canine renal transplantation [Zucker et al, 1996] also suggested the possibility of sequestration of IL-2 producing cells into the allograft or other immune organs like the spleen or bone marrow, quoting their previous work on mixed lymphocyte culture-reactive cells in canine renal transplants [Miller et al, 1971; Hattler et al, 1972]. Another possible reason to account for the fall in the overall level of peripheral IL-2 and IFN-γ gene expression could be the localisation of IL-2 and IFN-γ producing Th1 cells into lymphoid tissues where adhesion molecule upregulation in these cells and/or high endothelial venules results in the disappearance of these Th1 cells from the peripheral blood [Brockmeyer et al, 1993; Fuggle et al, 1993]. This sequestration hypothesis could be addressed by a study looking at a sequential and simultaneous sampling of peripheral blood and fine needle aspirations of the renal allografts so that the sequential cytokine gene expression profiles in peripheral blood may be correlated with changes in intragraft gene expression as well as with the clinical course of the patients following renal transplantation.

Overall, all the so-called Th2 cytokines studied in the project, namely, IL-4, IL-5, IL-10 and IL-13, showed more sequential changes following renal transplantation than the Th1 cytokines or CTL activation markers studied. A possible explanation for this may be that Th2 cells were more readily recirculated back into the peripheral blood following their "contact" with the allograft than the Th1 cells or the CTLs because they were not sequestered within the allograft or the other immune compartments. Consequently, the molecular imprints of the Th2 cells' "contact" with

the allograft were more prominently reflected by the sequential changes in the level of their cytokine gene expression in the peripheral blood. Another possible explanation for this observation is that Th2 cells may be more central in orchestrating the immune response to the transplanted allograft than either Th1 cells or CTLs and therefore the changes in Th2 alloreactivity were more marked than the other two groups of T cells.

Although peripheral IL-4 gene expression profiles showed the greatest number of sequential changes compared with all the other cytokines and CTL activation markers studied in the project (by having statistically significant sequential changes at almost all the time points in both rejectors and non-rejectors), our results suggest that the changes in the level of peripheral IL-4 gene expression can be explained more convincingly by the changes in the degree of immunosuppression rather than any clear differences between the rejectors and non-rejectors because of acute rejection. This suggests that sequential monitoring of peripheral IL-4 gene expression could potentially be useful as a method of assessing, at the molecular level, the degree of immunosuppression at the different time points following renal transplantation. However, monitoring of peripheral IL-4 gene expression was not able to distinguish between the rejectors from the non-rejectors.

IL-10 was the first Th2 cytokine studied in the project to show promise as a cytokine marker that could potentially distinguish the rejectors from the non-rejectors. Our study has shown that the peripheral IL-10 gene expression profile in the rejectors was distinctly different from that seen in the non-rejectors, although the profile in the rejectors following completion of anti-rejection therapy did not return to a pattern similar to that seen in the non-rejectors as one might have expected. Further follow-up peripheral blood samplings over a longer time period following the completion of anti-rejection therapy are required to determine whether the pattern of peripheral IL-10 gene expression following the anti-rejection therapy would return to the pattern seen in non-rejectors, i.e. at a level significantly above the pre-transplant baseline. This is an important aspect to be incorporated into the protocols for future studies as it would consolidate the evidence that IL-10 is indeed an important

peripheral cytokine marker which could distinguish between rejectors and non-rejectors.

IL-5 was the next Th2 cytokine that showed even more promise as a peripheral cytokine marker in distinguishing between rejectors and non-rejectors. Like IL-10, the post-transplant profile of IL-5 gene expression in the rejectors showed distinct differences from that seen in the non-rejectors, but unlike IL-10, the IL-5 profile returned to the pattern seen in non-rejectors following the successful completion of anti-rejection therapy, suggesting that IL-5 is a cytokine that most closely reflects the changes in T cell alloreactivity and thus most likely to be useful as a marker in the immunological monitoring of patients following renal transplantation. This finding is particularly exciting as it ties in with other intragraft studies [Martinez et al, 1992; Krams et al, 1992; Martinez et al, 1993a; Martinez et al, 1993b; Whitehead et al, 1993; Lang et al, 1995] associating IL-5 with acute allograft rejection and the non-classical pathway of acute allograft rejection involving eosinophils, the presence of eosinophils being a well known but unexplained phenomenon in transplantation immunology [reviewed in 1.5.5].

It has been discussed in the previous chapter that although IL-13 shares many biological activities with IL-4 and indeed may even share a common receptor or receptor component with IL-4 [as reviewed in 1.4.6], the peripheral T cell IL-13 gene expression profiles were found to be quite different from those for IL-4 in both non-rejectors and rejectors. Although the direction of the sequential changes seen for IL-13 was similar to IL-4 in the rejectors, the profiles for the non-rejectors were completely different for the two cytokines. It was this difference between the two cytokines that separated them and resulted in a clear difference between the rejectors' and non-rejectors' profiles for IL-13. Our study showed that the changes in the level of IL-13 gene expression relative to its pre-transplant baseline yielded data suggesting that peripheral IL-13 gene expression may be another promising marker in distinguishing between rejectors and non-rejectors, since clear differences in the level of IL-13 gene expression between the two groups of patients were seen at the time of acute rejection. This was a novel finding since the role of IL-13 in allograft rejection

has not been reported in the literature previously. Moreover, IL-13 is known to induce IgE synthesis and eosinophil adhesion to endothelial cells [reviewed in 1.5.6] and therefore its potential role in other T cell mediated processes like atopic diseases has been postulated [de Vries and Zurawski, 1995]. The results of our study demonstrating an increase in peripheral IL-13 gene expression at the time of acute rejection above the pre-transplant baseline and the subsequent return to the pre-transplant baseline following anti-rejection therapy, has tied in nicely with the our results for peripheral IL-5 gene expression profiles described in the previous paragraph.

Overall, both CTL activation markers, GrB and FasL, did not appear to have distinct post-transplant changes in their profiles to distinguish the rejectors from the non-rejectors.

The future

This project has demonstrated the experimental validity of sequential monitoring of peripheral blood T cell gene expression of patients following renal transplantation. It has also revealed a number of findings of potential immunological and clinical relevance which would not be readily detectable from single time point studies. In order that the technique may be applicable for possible clinical use in the future, it would need to be simplified further and the turnaround time shortened. Moreover, to allow the monitoring of gene expression of other immune cells in addition to the T cells, the use of the entire mononuclear cell fraction from peripheral blood instead of T cells alone would fulfil both objectives. The use of mononuclear cells instead of T cells only would enable the study of other important non-T cell-derived T cell growth factors like IL-12 and IL-15 which this project was not able to look into, and also increase the reliability of results from studying cytokines produced by both T cells and non-T cells like IL-10.

An increase in the frequency of peripheral blood sampling in the first fortnight following renal transplantation would undoubtedly improve the reliability in the use of these cytokine profiles in distinguishing between the potential rejectors from those unlikely to experience acute allograft rejection. A longer follow-up period than the one chosen in this project would allow a more detailed assessment of the subsequent profile of changes in cytokine gene expression following completion of anti-rejection therapy in those patients who do experience acute allograft rejection.

A parallel study looking into the sequential changes in intragraft cytokine gene expression at the same time as that occurring in the peripheral blood would allow the two profiles of changes to be correlated and enable a better understanding of the relationship between these immune cells sequestered into the renal allograft and those circulating in the peripheral blood. It could end the conflicting findings so prevalent in the literature by establishing the pattern of gene expression of the different cytokines that are important in the process underlying acute allograft rejection. However, the effectiveness of this study would inevitably be compromised by the limitation in the frequency of intragraft sampling for cytokine gene expression that would be acceptable to patients and clinicians alike since the sampling technique is invasive with potential complications.

The recent application of real-time detection of PCR products using fluorogenic probes (an oligonucleotide with both a reporter fluorescent dye and a quencher dye attached) [Lee et al, 1993; Livak et al, 1995] incorporated into the PCR itself for the quantification of cytokine gene expression in peripheral blood [Kruse et al, 1997] could potentially make immunological monitoring based on peripheral blood cytokine gene expression assay a clinical reality by considerably simplifying the technical aspect of the assay while at the same time shortening the turnaround time required for the assay to be performed. This so called 'TaqMan' assay is also advantageous in that all measurements are based on the exponential phase of the PCR, rather than its end-points. Theoretically, the amount of amplification required to produce a threshold amount of PCR product is much more closely related to the amount of starting material than it is to the final 'plateau' amount of end-point

product. Secondly, this technique can be rendered truly quantitative by standardizing against the total amount of mRNA starting material or the expression of a housekeeping gene.

Finally, with the recent surge in interest in the relationship of cytokine promoter gene polymorphisms - in particular, TNF-α and IL-10 - and acute allograft rejection [Sankaran et al, 1999], it will be important to investigate whether these polymorphisms do correlate with the cytokine gene expression profiles in renal transplant patients examined in this study.

Some of these points are being addressed by my successor as part of a new research study that is currently under way at the Wessex Renal and Transplant Unit in Portsmouth.

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APPENDIX 1

PROFORMA FOR THE CYTOKINE PROJECT

1. Recipient details: Code: _____

a) surname and firstname: _____

b) date of birth: ____/____/____

c) hospital number: _____

d) blood group: _____

e) tissue type: _____

f) CMV status: [ ] positive [ ] negative

g) cause of end-stage renal failure: _____

h) dialysis details: [ ] haemodialysis [ ] CAPD

2. Transplant details:

a) date of transplant (date of pre-transplant blood sampling): _____/_____/____

b) sampling: [ ] pre-HD [ ] post-HD, _____ hours post-HD

c) type - cadaveric / living related

d) side transplanted: R / L

e) primary / secondary transplant (state number of transplant): _____

f) donor blood group: _____

g) tissue type: _____

h) mismatch: ____:____:____ [A : B : DR]

i) CMV status: [ ] positive [ ] negative

j) % panel reactive antibodies: _____

3. Transplant function:

a) primary function? Y / N

b) biopsy? Y / N

c) histology result: [ ] cellular rejection [ ] vascular rejection
[ ] no rejection [ ] non-diagnostic
[ ] others _____

4. Date second blood sampling (day 2-3 post-transplant): _____/_____/____

a) blood indices: urea _____ creatinine _____ CyA level _____

WCC _____ Lymphocyte _____ Neutrophils _____

b) urine output: [ ] oliguric/anuric [ ] normal [ ] polyuric

c) any confirmed current/immediately recent infection:
[ ] UTI [ ] Chest infection
[ ] Fungal infection (oral/vaginal) [ ] CMV infection

d) pyrexia of unknown origin? Y / N

e) immunosuppression used: [ ] Cyclosporin [ ] Azathioprine
[ ] Prednisolone [ ] ATG

f) recent dialysis? Y / N If yes, _____ days ago

5. Date second blood sampling (day 5-7 post-transplant): _____/_____/____

a) blood indices: urea _____ creatinine _____ CyA level _____

WCC _____ Lymphocyte _____ Neutrophils _____

b) urine output: [ ] oliguric/anuric [ ] normal [ ] polyuric

c) any confirmed current/immediately recent infection:
[ ] UTI [ ] Chest infection
[ ] Fungal infection (oral/vaginal) [ ] CMV infection

d) pyrexia of unknown origin? Y / N

e) immunosuppression used: [ ] Cyclosporin [ ] Azathioprine
[ ] Prednisolone [ ] ATG

f) recent dialysis? Y / N If yes, _____ days ago

6. Date third blood sampling (day 10-14 post-transplant): _____/_____/____

a) blood indices: urea _____ creatinine _____ CyA level _____

WCC _____ Lymphocyte _____ Neutrophils _____

b) urine output: [ ] oliguric/anuric [ ] normal [ ] polyuric

c) any confirmed current/immediately recent infection:
[ ] UTI [ ] Chest infection
[ ] Fungal infection (oral/vaginal) [ ] CMV infection

d) pyrexia of unknown origin? Y / N

e) immunosuppression used: [ ] Cyclosporin [ ] Azathioprine
[ ] Prednisolone [ ] ATG

f) recent dialysis? Y / N If yes, _____ days ago

7. Date of fourth blood sampling (about day 30 post-transplant): _____/_____/____

a) blood indices: urea _____ creatinine _____ CyA level _____

WCC _____ Lymphocyte _____ Neutrophils _____

b) urine output: [ ] oliguric/anuric [ ] normal [ ] polyuric

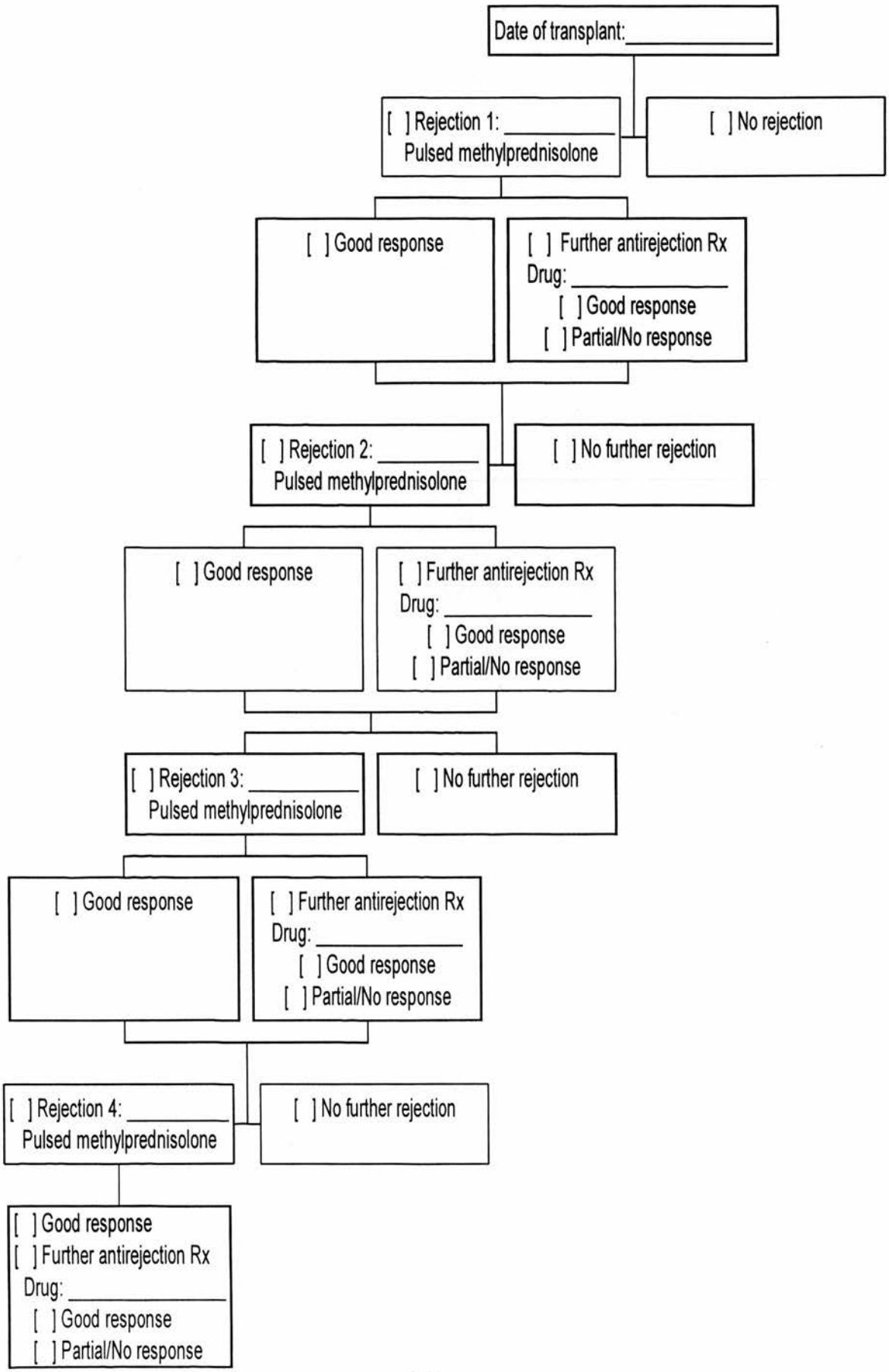
c) any confirmed current/immediately recent infection:
[ ] UTI [ ] Chest infection
[ ] Fungal infection (oral/vaginal) [ ] CMV infection

d) pyrexia of unknown origin? Y / N

e) immunosuppression used: [ ] Cyclosporin [ ] Azathioprine
[ ] Prednisolone [ ] ATG

f) recent dialysis? Y / N If yes, _____ days ago

Transplant flow chart



Follow-up Proforma

Name: _____

1. a) date: _____/_____/____ days post-transplant: _____

   b) blood indices: urea _____ creatinine _____ CyA level _____

   WCC _____ Lymphocyte _____ Neutrophils _____

   c) urine output: [ ] oliguric/anuric [ ] normal [ ] polyuric

   d) any confirmed current/immediately recent infection:
   [ ] UTI [ ] Chest infection
   [ ] Fungal infection (oral/vaginal) [ ] CMV infection

   e) pyrexia of unknown origin? Y / N

   f) immunosuppression used: [ ] Cyclosporin [ ] Azathioprine
   [ ] Prednisolone [ ] ATG

2. a) date: _____/_____/____ days post-transplant: _____

   b) blood indices: urea _____ creatinine _____ CyA level _____

   WCC _____ Lymphocyte _____ Neutrophils _____

   c) urine output: [ ] oliguric/anuric [ ] normal [ ] polyuric

   d) any confirmed current/immediately recent infection:
   [ ] UTI [ ] Chest infection
   [ ] Fungal infection (oral/vaginal) [ ] CMV infection

   e) pyrexia of unknown origin? Y / N

   f) immunosuppression used: [ ] Cyclosporin [ ] Azathioprine
   [ ] Prednisolone [ ] ATG

3. a) date: _____/_____/____ days post-transplant: _____

   b) blood indices: urea _____ creatinine _____ CyA level _____

   WCC _____ Lymphocyte _____ Neutrophils _____

   c) urine output: [ ] oliguric/anuric [ ] normal [ ] polyuric

   d) any confirmed current/immediately recent infection:
   [ ] UTI [ ] Chest infection
   [ ] Fungal infection (oral/vaginal) [ ] CMV infection

   e) pyrexia of unknown origin? Y / N

   f) immunosuppression used: [ ] Cyclosporin [ ] Azathioprine
   [ ] Prednisolone [ ] ATG

REJECTION DETAILS

Name: _____

Rejection episode 1:

a) date blood sampled prior to anti-rejection therapy: _____/_____/____

b) blood indices: urea _____ creatinine _____ CyA level _____

c) recent haemodialysis dialysis? Y / N If yes, _____ days ago

d) biopsy? Y / N

e) histology: [ ] cellular rejection [ ] vascular rejection
[ ] no rejection [ ] non-diagnostic
[ ] others _____

f) pulsed methylprednisolone? Y / N

date blood sampled following course of anti-rejection therapy: _____/_____/____

g) anti-thymocyte globulin therapy? Y / N

If yes, dosage: ____ mg; total number of doses: _____ over ____days

date blood sampled following course of anti-rejection therapy: _____/_____/____

h) response: [ ] complete success (full restoration of graft function)
[ ] partial success (partial restoration of graft function)
[ ] failure with loss of graft function

i) date blood sampled 7-10 days following end of anti-rejection therapy: _____/_____/____

Rejection episode 2:

a) date blood sampled prior to anti-rejection therapy: _____/_____/____

b) blood indices: urea _____ creatinine _____ CyA level _____

c) recent haemodialysis dialysis? Y / N If yes, _____ days ago

d) biopsy? Y / N

e) histology: [ ] cellular rejection [ ] vascular rejection
[ ] no rejection [ ] non-diagnostic
[ ] others _____

f) pulsed methylprednisolone? Y / N

date blood sampled following course of anti-rejection therapy: _____/_____/____

g) anti-thymocyte globulin therapy? Y / N

If yes, dosage: ____ mg; total number of doses: _____ over ____days

date blood sampled following course of anti-rejection therapy: _____/_____/____

h) response: [ ] complete success (full restoration of graft function)
[ ] partial success (partial restoration of graft function)
[ ] failure with loss of graft function

i) date blood sampled 7-10 days following end of anti-rejection therapy: _____/_____/____

Rejection episode 3:

a) date blood sampled prior to anti-rejection therapy: _____/_____/____

b) blood indices: urea _____ creatinine _____ CyA level _____

c) recent haemodialysis dialysis? Y / N If yes, _____ days ago

d) biopsy? Y / N

e) histology: [ ] cellular rejection [ ] vascular rejection
[ ] no rejection [ ] non-diagnostic
[ ] others _____

f) pulsed methylprednisolone? Y / N

date blood sampled following course of anti-rejection therapy: _____/_____/____

g) anti-thymocyte globulin therapy? Y / N

If yes, dosage: ____ mg; total number of doses: _____ over ____days

date blood sampled following course of anti-rejection therapy: _____/_____/____

h) response: [ ] complete success (full restoration of graft function)
[ ] partial success (partial restoration of graft function)
[ ] failure with loss of graft function

i) date blood sampled 7-10 days following end of anti-rejection therapy: _____/_____/____

Rejection episode 4:

a) date blood sampled prior to anti-rejection therapy: _____/_____/____

b) blood indices: urea _____ creatinine _____ CyA level _____

c) recent haemodialysis dialysis? Y / N If yes, _____ days ago

d) biopsy? Y / N

e) histology: [ ] cellular rejection [ ] vascular rejection
[ ] no rejection [ ] non-diagnostic
[ ] others _____

f) pulsed methylprednisolone? Y / N

date blood sampled following course of anti-rejection therapy: _____/_____/____

g) anti-thymocyte globulin therapy? Y / N

If yes, dosage: ____ mg; total number of doses: _____ over ____days

date blood sampled following course of anti-rejection therapy: _____/_____/____

h) response: [ ] complete success (full restoration of graft function)
[ ] partial success (partial restoration of graft function)
[ ] failure with loss of graft function

i) date blood sampled 7-10 days following end of anti-rejection therapy: _____/_____/____

APPENDIX 2

Portsmouth and
South East Hampshire

Health Commission

Finchdean House
Milton Road
Portsmouth PO3 6DP

Switchboard: (01705) 838340
Central Fax: (01705) 733292

Direct Line (01705) 835092
Fax Number (01705) 733292

Our Ref RJT/VS/recua.wcm Your Ref

26 March 1996

Mr Lam Chin Tan
Clinical Research Fellow
Flat 6
York House
Queen Alexandra Hospital
Cosham
Portsmouth
Hants

Dear Mr Tan

REC Prop No: 03/96/353 Cytokine Gene Expression in Renal Transplantation

This is to confirm that the Research Ethics Committee has approved the above study. Approval for the study is only granted until the 1st October 1997, if your study continues after this date further ethics committee approval will be required.

I remind you that you stated patients will not be participating in any other clinical trials. If there is any change, you are obliged to draw this to the Committee's attention.

The Ethics Committee will require a copy of the completed study for its records, you are therefore requested to submit a copy of the completed study to the address above. In addition the Committee must be informed of any untoward or adverse events which occur during the course of the study.

The ethics committee must also be informed of and approve, any proposed amendments to your initial application.

If you have any further questions please do not hesitate to contact me.

Yours sincerely

Dr E Wozniak
Chairman - Research Ethics Committee

APPENDIX 3

Mr Saml Sadek, PhD, FRCS - Head
Mr Martin Wise, MD, FRCS
Miss Anne M Walters, MD, FRCS

Transplant Surgery

PORTSMOUTH HOSPITALS NHS TRUST

Wessex Renal and Transplant Unit
St Mary's Hospital
Portsmouth PO3 6AD
☎ Tel 02392 286000 Ext 3120
Fax 02392 866108

Consent Form For Participation in Research Project

I, _____, having read the information leaflet about the research project, hereby agrees to have bloods taken from me periodically for analysis.

I understand that the research project will not have anything whatsover to do with my care.

I also understand that I can withdraw my participation in the research project at any time without my care being affected in any way.

Signed: _____ Date: _____

APPENDIX 4

Mr Sami Sadek, PhD, FRCS - Head
Mr Martin Wise, MD, FRCS
Miss Anne M Walters, MD, FRCS

Transplant Surgery

PORTSMOUTH HOSPITALS NHS TRUST

Wessex Renal and Transplant Unit
St Mary's Hospital
Portsmouth PO3 6AD
Tel 02392 286000 Ext 3120
Fax 02392 866108

Information Leaflet For The Research Project

The Wessex Regional Renal and Transplant Unit at St Mary's Hospital, Portsmouth is currently researching into why the defence mechanism of your body (called the immune system) may reject your kidney transplant despite being suppressed by powerful drugs.

We hope we could enlist your help in our project. The research will not benefit your care directly but it may help us to improve the care that we can give to patients receiving a new kidney in the future by increasing our knowledge of the body's defence mechanism.

It will involve us taking some blood specimens from you periodically from the time you receive your new kidney transplant to 6 weeks following the operation.

We emphasize that you are under no obligation to participate in the research project and your care will not be compromised in any way should you choose not to participate in the research project. You are also at liberty to withdraw your participation in the research project at any time.

We sincerely hope that you will help us in our efforts to improve the results of kidney transplantation.

APPENDIX 5 - Sampling Codes for Appendices 6 to 11

Protocol sampling time points

Codes	Sampling Time Points
/PT	Pre-transplant
/2	Early post-transplant
/3	1 week post-transplant
/4	2 weeks post-transplant
/5	4 weeks post-transplant

Additional Samples For Rejectors

For the rejectors, note the following:

a) the number pre-fixing the sampling codes refer to the rejection episode number, i.e. 1 meaning first rejection episode, etc.

b) the letter "a" following the post-anti-rejection therapy sampling code means that the sample was taken following antibody anti-rejection therapy.

Codes	Sampling Time Points
/PR	Pre-anti-rejection therapy sample
/R1	Early post-anti-rejection therapy sample (usually post-steroid pulse)
/R2	1 week post-anti-rejection therapy sample
/R3	4 weeks post-anti-rejection therapy sample

Notes on calculation of percentage change in appendices 8 and 9:

PCR ELISA reading for each cytokine at pre-transplant time point (/PT) = Y
PCR ELISA reading for each cytokine at time point x = X
where /x=any post-Tx time point.
Percentage change at time point x = [Exponential (X) /Exponential (Y)] x 100

Note that the PCR ELISA readings are in natural logarithmic scale.

APPENDIX 6 - Cell Separation Data

				WHOLE BLOOD					SEPARATED CELLS			
Date	Subject	No.	Sample	LY	MO	Vol.	No.	% MO	MNC	Vol.	No.	% Loss
5/14/96	EP	001	/PT	1.6	0.4	13.5	27.0	20.0	0.6	15.0	9.0	66.7
5/16/96	EP	001	/2	0.6	0.9	13.5	20.3	60.0	0.3	15.0	4.5	77.8
5/20/96	EP	001	/3	1.4	0.1	13.5	20.3	6.7	0.5	17.0	8.5	58.0
5/24/96	EP	001	/4	1.6	0.5	13.5	28.4	23.8	0.8	15.0	12.0	57.7
5/26/96	FO	003	/PT	0.9	0.6	13.5	20.3	40.0	0.3	15.0	4.5	77.8
5/27/96	PH	005	/PT	2.8	0.6	10.8	36.7	17.6	1.2	15.0	18.0	51.0
5/29/96	BR	006	/PT	2.0	0.6	13.5	35.1	23.1	1.0	15.0	15.0	57.3
5/29/96	FO	003	/2	0.6	0.5	13.5	14.9	45.5	0.2	15.0	3.0	79.8
5/29/96	PG	007	/PT	0.9	0.7	13.5	21.6	43.8	0.4	15.0	6.0	72.2
5/29/96	PH	005	/2	1.0	1.2	13.5	29.7	54.5	0.7	15.0	10.5	64.6
5/31/96	BR	006	/2	0.7	0.1	9.0	7.2	12.5	0.3	15.0	4.5	37.5
5/31/96	FO	003	/3	0.6	1.1	9.0	15.3	64.7	0.2	15.0	3.0	80.4
5/31/96	PG	007	/2	0.4	0.5	13.5	12.2	55.6	0.2	15.0	3.0	75.3
6/3/96	PH	005	/3	1.9	2.1	13.5	54.0	52.5	NR	15.0	NR	NR
6/5/96	BR	006	/3	1.9	0.9	13.5	37.8	32.1	1.3	15.0	19.5	48.4
6/5/96	JM	008	/PT	1.6	0.5	13.5	28.4	23.8	1.0	15.0	15.0	47.1
6/5/96	PG	007	/3	0.7	0.6	13.5	17.6	46.2	0.3	15.0	4.5	74.4
6/7/96	FO	003	/4	1.0	0.6	13.5	21.6	37.5	0.4	15.0	6.0	72.2
6/7/96	JM	008	/2	0.5	0.3	13.5	10.8	37.5	0.2	15.0	3.0	72.2
6/7/96	PH	005	/4	3.7	1.4	13.5	68.9	27.5	2.8	15.0	42.0	39.0
6/10/96	BR	006	/1R1	1.2	0.2	13.5	18.9	14.3	1.0	15.0	15.0	20.6
6/10/96	EP	001	/5	1.2	0.1	13.5	17.6	7.7	0.6	15.0	9.0	48.7
6/10/96	PG	007	/4	0.6	0.3	13.5	12.2	33.3	0.4	15.0	6.0	50.6
6/11/96	EJ	010	/PT	0.9	0.3	9.0	10.8	25.0	0.5	15.0	7.5	30.6
6/11/96	JM	008	/3	0.7	0.4	13.5	14.9	36.4	0.5	15.0	7.5	49.5
6/11/96	JW	009	/PT	0.9	0.4	13.5	17.6	30.8	0.6	15.0	9.0	48.7
6/14/96	EJ	010	/2	0.7	0.6	13.5	17.6	46.2	0.3	15.0	4.5	74.4
6/14/96	JW	009	/2	0.4	0.6	13.5	13.5	60.0	0.3	15.0	4.5	66.7
6/17/96	BR	006	/1R2	1.2	0.6	13.5	24.3	33.3	0.9	15.0	13.5	44.4
6/17/96	JM	008	/4	0.7	0.7	13.5	18.9	50.0	0.4	15.0	6.0	68.3
6/18/96	EJ	010	/3	1.5	0.6	13.3	27.9	28.6	0.9	15.0	13.5	51.7
6/18/96	JW	009	/3	1.4	0.6	13.3	26.6	30.0	0.9	15.0	13.5	49.2
6/18/96	SK	011	/PT	0.9	0.6	13.5	20.3	40.0	0.6	15.0	9.0	55.6
6/24/96	FO	003	/5	0.3	0.8	13.5	14.9	72.7	0.3	15.0	4.5	69.7
6/24/96	JW	009	/4	1.4	0.9	13.5	31.1	39.1	1.0	15.0	15.0	51.7
6/25/96	BR	006	/2R1	1.0	0.3	13.5	17.6	23.1	0.6	15.0	9.0	48.7
6/25/96	EJ	010	/4	1.3	0.8	13.5	28.4	38.1	0.8	15.0	12.0	57.7
6/25/96	JM	008	/1R1	0.3	0.2	13.5	6.8	40.0	0.1	15.0	1.5	77.8
6/25/96	PH	005	/1PR	1.2	1.0	13.5	29.7	45.5	0.9	15.0	13.5	54.5
6/28/96	PH	005	/1R1	NR	NR	13.5	NR	NR	0.3	15.0	4.5	NR
6/28/96	SK	011	/2	2.0	0.7	13.5	36.5	25.9	1.3	15.0	19.5	46.5

APPENDIX 6 - Cell Separation Data

				WHOLE BLOOD					SEPARATED CELLS			
Date	Subject	No.	Sample	LY	MO	Vol.	No.	% MO	MNC	Vol.	No.	% Loss
7/1/96	SK	011	/3	4.4	0.9	13.5	71.6	17.0	3.0	15.0	45.0	37.1
7/1/96	BR	006	/2R2	1.1	0.1	13.5	16.2	8.3	0.7	15.0	10.5	35.2
7/1/96	JM	008	/1R2	0.3	0.3	13.5	8.1	50.0	0.3	15.0	4.5	44.4
7/2/96	EJ	010	/1PR	0.5	0.5	13.5	13.5	50.0	0.4	15.0	6.0	55.6
7/2/96	PG	007	/5	0.7	0.7	13.5	18.9	50.0	0.4	15.0	6.0	68.3
7/5/96	PH	005	/1R2	1.1	1.0	13.5	28.4	47.6	0.8	15.0	12.0	57.7
7/5/96	EJ	010	/1R1	0.3	0.2	13.5	6.8	40.0	0.2	15.0	3.0	55.6
7/8/96	SK	011	/4	3.6	1.3	13.3	65.2	26.5	3.0	15.0	45.0	30.9
7/8/96	DR	013	/PT	1.3	0.5	13.5	24.3	27.8	0.7	15.0	10.5	56.8
7/10/96	TG	014	/PT	2.8	0.8	13.5	48.6	22.2	1.8	15.0	27.0	44.4
7/10/96	MP	015	/PT	2.1	0.8	13.5	39.2	27.6	0.7	15.0	10.5	73.2
7/12/96	DR	013	/2	1.3	0.7	9.0	18.0	35.0	0.5	15.0	7.5	58.3
7/12/96	TG	014	/2	1.2	1.0	8.1	17.8	45.5	0.4	15.0	6.0	66.3
7/12/96	MP	015	/2	1.1	1.0	13.5	28.4	47.6	0.8	15.0	12.0	57.7
7/15/96	DR	013	/3	1.6	0.9	13.5	33.8	36.0	1.0	15.0	15.0	55.6
7/15/96	JW	009	/5	1.0	0.5	13.5	20.3	33.3	0.5	15.0	7.5	63.0
7/17/96	EJ	010	/1R2	0.3	0.5	13.5	10.8	62.5	0.2	15.0	3.0	72.2
7/17/96	TG	014	/3	1.3	1.6	13.5	39.2	55.2	0.9	15.0	13.5	65.5
7/17/96	MP	015	/3	1.9	1.2	13.5	41.9	38.7	1.2	15.0	18.0	57.0
7/22/96	TG	014	/1R1	1.6	0.8	13.3	31.9	33.3	1.0	15.0	15.0	53.0
7/22/96	MP	015	/4	2.5	1.4	13.5	52.7	35.9	1.9	15.0	28.5	45.9
7/22/96	SK	011	/5	2.9	1.0	13.4	52.3	25.6	2.3	15.0	34.5	34.0
7/23/96	DR	013	/4	1.0	1.4	13.5	32.4	58.3	0.6	16.0	9.6	70.4
7/29/96	TG	014	/1R2	1.0	0.5	13.4	20.1	33.3	0.6	15.0	9.0	55.2
8/5/96	DR	013	/5	1.1	0.7	13.5	24.3	38.9	0.7	16.0	11.2	53.9
8/5/96	MP	015	/1R1	1.7	0.3	13.5	27.0	15.0	1.1	17.0	18.7	30.7
8/16/96	MP	015	/1R2	0.2	0.2	19.0	7.6	50.0	0.2	15.0	3.0	60.5
10/2/96	BT	016	/PT	1.5	0.5	13.5	27.0	25.0	0.8	15.0	12.0	55.6
10/4/96	BT	016	/2	0.5	1.2	18.0	30.6	70.6	NR	16.0	NR	NR
10/7/96	BT	016	/3	0.8	0.9	16.0	27.2	52.9	0.5	15.0	7.5	72.4
10/7/96	JS	017	/PT	1.9	0.7	16.0	41.6	26.9	1.3	15.0	19.5	53.1
10/11/96	JS	017	/2	2.0	1.4	16.0	54.4	41.2	NR	15.0	NR	NR
10/14/96	BT	016	/4	1.3	0.9	14.0	30.8	40.9	0.8	15.0	12.0	61.0
10/14/96	JS	017	/3	1.8	1.2	15.0	45.0	40.0	1.0	15.0	15.0	66.7
10/15/96	PC	018	/PT	1.2	0.3	17.0	25.5	20.0	1.0	15.0	15.0	41.2
10/18/96	PC	018	/2	1.2	0.6	17.0	30.6	33.3	0.9	15.0	13.5	55.9
10/21/96	PC	018	/3	1.6	0.2	16.0	28.8	11.1	0.9	15.0	13.5	53.1
10/21/96	JS	017	/4	NR	NR	16.0	NR	NR	2.9	15.0	43.5	NR
10/21/96	EC	019	/PT	1.3	0.4	16.0	27.2	23.5	1.5	15.0	22.5	17.3
10/25/96	EC	019	/2	NR	NR	16.0	NR	NR	0.3	16.0	4.8	NR
10/28/96	PC	018	/4	1.6	0.7	17.0	39.1	30.4	1.5	15.0	22.5	42.5

APPENDIX 6 - Cell Separation Data

				WHOLE BLOOD					SEPARATED CELLS			
Date	Subject	No.	Sample	LY	MO	Vol.	No.	% MO	MNC	Vol.	No.	% Loss
10/28/96	EC	019	/3	0.9	1.6	16.0	40.0	64.0	0.6	15.0	9.0	77.5
10/29/96	RF	020	/PT	0.9	0.6	17.0	25.5	40.0	0.6	15.0	9.0	64.7
10/29/96	AB	021	/PT	0.6	0.4	17.0	17.0	40.0	0.4	15.0	6.0	64.7
11/1/96	EC	019	/1R1	0.8	0.8	17.0	27.2	50.0	0.7	16.0	11.2	58.8
11/1/96	RF	020	/2	NR	NR	15.0	NR	NR	0.8	16.0	12.8	NR
11/1/96	AB	021	/2	0.5	0.5	15.0	15.0	50.0	0.3	15.0	4.5	70.0
11/4/96	RF	020	/3	1.9	0.9	14.0	39.2	32.1	1.1	15.0	16.5	57.9
11/4/96	AB	021	/3	0.5	0.6	16.0	17.6	54.5	0.4	15.0	6.0	65.9
11/6/96	RE	022	/PT	1.1	0.5	16.0	25.6	31.3	0.8	15.0	12.0	53.1
11/8/96	RE	022	/2	1.6	0.9	14.5	36.3	36.0	0.8	15.0	12.0	66.9
11/8/96	EC	019	/1R2	1.8	1.3	16.0	49.6	41.9	1.1	15.0	16.5	66.7
11/11/96	BT	016	/5	0.8	1.0	14.0	25.2	55.6	0.9	15.0	13.5	46.4
11/11/96	JS	017	/5	3.1	1.0	14.0	57.4	24.4	2.1	15.0	31.5	45.1
11/11/96	PC	018	/5	1.2	0.5	15.0	25.5	29.4	0.8	15.0	12.0	52.9
11/11/96	RF	020	/4	1.7	0.8	15.5	38.8	32.0	1.3	15.0	19.5	49.7
11/11/96	AB	021	/4	0.8	0.6	15.0	21.0	42.9	0.1	15.0	1.5	92.9
11/12/96	RE	022	/3	2.5	0.6	16.0	49.6	19.4	1.8	15.0	27.0	45.6
11/12/96	NH	023	/PT	0.9	0.7	16.0	25.6	43.8	0.8	15.0	12.0	53.1
11/12/96	BM	024	/PT	2.1	1.0	16.0	49.6	32.3	1.5	15.0	22.5	54.6
11/14/96	VH	025	/PT	0.9	0.6	15.5	23.3	40.0	0.6	15.0	9.0	61.3
11/15/96	NH	023	/2	0.8	0.5	16.0	20.8	38.5	0.7	15.0	10.5	49.5
11/15/96	BM	024	/2	1.8	1.4	16.0	51.2	43.8	1.4	16.0	22.4	56.3
11/18/96	RE	022	/4	1.7	0.3	17.0	34.0	15.0	1.2	16.0	19.2	43.5
11/18/96	NH	023	/3	1.1	0.6	16.0	27.2	35.3	0.9	15.0	13.5	50.4
11/18/96	BM	024	/3	1.5	0.9	16.0	38.4	37.5	1.1	15.0	16.5	57.0
11/18/96	VH	025	/2	0.6	0.9	14.0	21.0	60.0	0.5	16.0	8.0	61.9
11/19/96	EC	019	/2PR	2.1	0.8	15.5	45.0	27.6	1.0	15.0	15.0	66.6
11/19/96	AB	021	/1PR	0.6	1.0	16.0	25.6	62.5	0.4	15.0	6.0	76.6
11/20/96	VH	025	/3	0.8	1.1	16.0	30.4	57.9	0.5	15.0	7.5	75.3
11/22/96	AB	021	/1R1	0.3	0.4	16.0	11.2	57.1	0.2	15.0	3.0	73.2
11/22/96	NH	023	/1PR	1.1	0.6	16.0	27.2	35.3	1.0	15.0	15.0	44.9
11/25/96	NH	023	/1R1	0.6	NR	15.5	NR	NR	0.4	15.0	6.0	NR
11/25/96	BM	024	/4	1.7	NR	16.0	NR	NR	1.0	15.0	15.0	NR
11/25/96	VH	025	/1R1	0.8	NR	16.0	NR	NR	0.7	15.0	10.5	NR
11/25/96	RF	020	/5	3.0	0.4	16.5	56.1	11.8	2.2	15.0	33.0	41.2
12/2/96	NH	023	/2R1	0.6	NR	16.0	NR	NR	0.4	15.0	6.0	NR
12/2/96	AB	021	/1R2	1.2	0.5	15.0	25.5	29.4	0.8	15.0	12.0	52.9
12/2/96	VH	025	/1R2	NR	NR	16.5	NR	NR	0.9	15.0	13.5	NR
12/9/96	BM	024	/5	1.5	0.2	14.5	24.7	11.8	0.7	15.0	10.5	57.4
12/9/96	RE	022	/5	0.8	0.2	12.0	12.0	20.0	0.3	15.0	4.5	62.5
12/9/96	NH	023	/2R2	0.3	0.1	15.5	6.2	25.0	0.2	15.0	3.0	51.6

APPENDIX 6 - Cell Separation Data

				WHOLE BLOOD					SEPARATED CELLS			
Date	Subject	No.	Sample	LY	MO	Vol.	No.	% MO	MNC	Vol.	No.	% Loss
12/11/96	SM	026	/PT	1.5	0.6	15.5	32.6	28.6	0.8	15.0	12.0	63.1
12/13/96	SM	026	/2	1.3	0.8	16.0	33.6	NR	NR	16.0	NR	NR
12/17/96	SM	026	/3	2.4	0.8	16.0	51.2	25.0	1.4	15.0	21.0	59.0
12/17/96	VH	025	/1R3	1.2	0.9	15.5	32.6	42.9	0.8	15.0	12.0	63.1
12/19/96	AB	021	/1R3	1.5	0.7	17.0	37.4	31.8	0.8	15.0	12.0	67.9
12/23/96	NH	023	/2R3	1.1	0.3	13.0	18.2	21.4	0.6	15.0	9.0	50.5
12/23/96	SM	026	/4	2.8	1.2	12.5	50.0	30.0	1.3	15.0	19.5	61.0
1/13/97	SM	026	/5	2.3	0.8	16.5	51.2	25.8	1.4	15.0	21.0	58.9
1/22/97	KB	027	/PT	2.3	0.6	15.5	45.0	20.7	1.5	15.0	22.5	49.9
1/24/97	KB	027	/2	1.4	1.0	15.5	37.2	41.7	0.5	15.0	7.5	79.8
1/29/97	KB	027	/3	4.0	NR	13.0	NR	NR	2.1	15.0	31.5	NR
2/3/97	KB	027	/4	2.6	1.4	14.0	56.0	35.0	1.5	15.0	22.5	59.8
2/3/97	JH	029	/PT	2.1	0.7	16.5	46.2	25.0	1.4	15.0	21.0	54.5
2/4/97	CA	030	/PT	1.8	0.8	16.0	41.6	30.8	1.4	15.0	21.0	49.5
2/4/97	RW	031	/PT	1.5	0.9	16.0	38.4	37.5	0.8	15.0	12.0	68.8
2/5/97	JH	029	/2	1.0	1.0	16.0	32.0	50.0	0.6	15.0	9.0	71.9
2/5/97	GK	032	/PT	1.6	0.4	16.0	32.0	20.0	1.0	15.0	15.0	53.1
2/7/97	CA	030	/2	1.9	1.1	15.0	45.0	36.7	0.9	15.0	13.5	70.0
2/7/97	RW	031	/2	0.5	0.5	15.5	15.5	50.0	0.3	15.0	4.5	71.0
2/7/97	GK	032	/2	2.1	0.9	16.0	48.0	30.0	1.4	15.0	21.0	56.3
2/10/97	JH	029	/3	1.7	1.2	15.5	45.0	41.4	1.3	15.0	19.5	56.6
2/10/97	AA	033	/PT	1.0	0.4	15.5	21.7	28.6	0.5	15.0	7.5	65.4
2/11/97	CA	030	/3	3.3	0.2	12.5	43.8	5.7	1.8	15.0	27.0	38.3
2/11/97	RW	031	/3	2.4	0.2	10.5	27.3	7.7	1.0	15.0	15.0	45.1
2/12/97	GK	032	/3	3.7	1.0	14.5	68.2	21.3	2.4	15.0	36.0	47.2
2/12/97	AA	033	/2	0.6	0.2	16.0	12.8	25.0	0.4	15.0	6.0	53.1
2/14/97	JH	029	/4	1.7	1.1	14.5	40.6	39.3	1.3	15.0	19.5	52.0
2/17/97	CA	030	/4	3.0	1.0	16.5	66.0	25.0	2.1	15.0	31.5	52.3
2/17/97	RW	031	/4	1.1	0.5	16.5	26.4	31.3	0.6	15.0	9.0	65.9
2/17/97	GK	032	/4	3.5	0.9	15.5	68.2	20.5	2.4	15.0	36.0	47.2
2/17/97	AA	033	/3	1.6	0.6	14.5	31.9	27.3	0.9	15.0	13.5	57.7
2/19/97	JH	029	/4	1.7	0.4	16.5	34.7	19.0	0.8	15.0	12.0	65.4
2/19/97	NR	034	/PT	1.4	0.2	17.0	27.2	12.5	1.0	16.0	16.0	41.2
2/21/97	AA	033	/4	1.9	0.7	16.0	41.6	26.9	1.2	15.0	18.0	56.7
2/21/97	NR	034	/2	1.5	0.4	16.0	30.4	21.1	0.8	15.0	12.0	60.5
2/21/97	LH	035	/PT	0.7	0.2	16.0	14.4	22.2	0.4	15.0	6.0	58.3
2/24/97	LH	035	/2	0.3	0.1	16.0	6.4	25.0	0.3	15.0	4.5	29.7
2/24/97	DK	036	/PT	1.4	0.6	14.0	28.0	30.0	0.7	15.0	10.5	62.5
2/24/97	KB	037	/PT	1.1	0.5	16.0	25.6	31.3	0.7	15.0	10.5	59.0
2/27/97	LH	035	/3	0.4	NR	16.5	NR	NR	0.2	15.0	3.0	NR
2/27/97	DK	036	/2	1.5	0.8	16.5	38.0	34.8	1.0	15.0	15.0	60.5

APPENDIX 6 - Cell Separation Data

				WHOLE BLOOD					SEPARATED CELLS			
Date	Subject	No.	Sample	LY	MO	Vol.	No.	% MO	MNC	Vol.	No.	% Loss
2/27/97	KB	037	/2	1.0	1.0	15.5	31.0	50.0	0.6	15.0	9.0	71.0
3/3/97	DK	036	/3	1.9	1.2	16.0	49.6	38.7	1.2	15.0	18.0	63.7
3/3/97	KB	037	/3	1.7	1.0	14.5	39.2	37.0	1.2	15.0	18.0	54.0
3/3/97	RW	031	/5	0.6	0.5	13.5	14.9	45.5	0.3	15.0	4.5	69.7
3/3/97	CA	030	/5	1.6	0.6	10.0	22.0	27.3	0.6	15.0	9.0	59.1
3/5/97	JH	029	/1R1a	0.1	0.0	16.0	1.6	0.0	0.1	15.0	1.5	6.3
3/5/97	IL	038	/PT	1.7	0.2	15.0	28.5	10.5	0.9	15.0	13.5	52.6
3/6/97	KB	027	/5	1.1	1.1	14.0	30.8	50.0	0.7	15.0	10.5	65.9
3/6/97	GK	032	/5	2.6	0.5	14.0	43.4	16.1	1.4	15.0	21.0	51.6
3/7/97	NR	034	/1R1	0.4	1.2	16.5	26.4	75.0	0.2	15.0	3.0	88.6
3/7/97	LH	035	/4	0.6	0.3	15.0	13.5	33.3	0.4	15.0	6.0	55.6
3/7/97	IL	038	/2	0.4	0.2	16.0	9.6	33.3	0.2	15.0	3.0	68.8
3/10/97	AA	033	/5	2.2	0.6	13.0	36.4	21.4	0.9	15.0	13.5	62.9
3/10/97	DK	036	/4	2.9	1.0	17.0	66.3	25.6	1.7	15.0	25.5	61.5
3/10/97	KB	037	/4	1.5	0.8	17.0	39.1	34.8	1.1	15.0	16.5	57.8
3/13/97	JH	029	/1R2	1.0	0.8	16.5	29.7	44.4	0.7	15.0	10.5	64.6
3/13/97	NR	034	/1R2	0.4	0.3	17.0	11.9	42.9	0.1	15.0	1.5	87.4
3/13/97	IL	038	/3	0.8	0.3	16.5	18.2	27.3	0.4	15.0	6.0	66.9
3/17/97	IL	038	/4	1.9	0.6	17.0	42.5	24.0	0.5	15.0	7.5	82.4
3/17/97	NR	034	/1R2	0.3	0.6	16.5	14.9	66.7	0.1	15.0	1.5	89.9
3/19/97	JR	039	/PT	1.7	0.4	14.0	29.4	19.0	1.0	15.0	15.0	49.0
3/21/97	MS	040	/PT	1.7	0.7	16.0	38.4	29.2	0.8	15.0	12.0	68.8
3/24/97	MS	040	/2	1.5	0.8	16.0	36.8	34.8	0.7	15.0	10.5	71.5
3/24/97	DK	036	/5	2.4	1.1	14.4	50.4	31.4	1.2	15.0	18.0	64.3
3/24/97	KB	037	/5	1.1	0.9	16.0	32.0	45.0	0.6	15.0	9.0	71.9
3/24/97	LH	035	/5	0.5	0.2	16.0	11.2	28.6	0.3	15.0	4.5	59.8
3/27/97	MS	040	/3	1.7	0.8	16.5	41.3	32.0	1.0	15.0	15.0	63.6
4/4/97	JR	039	/2	1.1	0.5	17.5	28.0	31.3	0.8	15.0	12.0	57.1
4/4/97	MS	040	/4	1.3	0.6	16.0	30.4	31.6	0.7	15.0	10.5	65.5
4/7/97	JH	029	/1R3	1.4	0.3	16.0	27.2	17.6	0.5	15.0	7.5	72.4
4/7/97	IL	038	/5	1.8	0.6	16.0	38.4	25.0	1.4	15.0	21.0	45.3
4/7/97	NR	034	/1R3	0.2	0.4	15.5	9.3	66.7	0.1	15.0	1.5	83.9
4/9/97	JR	039	/3	2.9	0.7	16.5	59.4	19.4	2.1	15.0	31.5	47.0
4/14/97	JR	039	/4	2.9	0.8	17.5	64.8	21.6	1.8	15.0	27.0	58.3
4/21/97	MS	040	/5	0.6	0.6	16.0	19.2	50.0	0.3	15.0	4.5	76.6
4/24/97	IT	041	/PT	2.2	1.0	16.5	52.8	31.3	1.5	15.0	22.5	57.4
4/28/97	IT	041	/2	0.9	0.9	17.0	30.6	50.0	0.5	15.0	7.5	75.5
4/30/97	IT	041	/3	1.4	1.1	16.5	41.3	44.0	0.8	15.0	12.0	70.9
4/30/97	CB	042	/PT	1.8	0.4	17.0	37.4	18.2	1.0	15.0	15.0	59.9
5/2/97	CB	042	/2	1.4	0.3	14.5	24.7	17.6	0.7	15.0	10.5	57.4
5/5/97	EK	043	/PT	1.5	0.3	16.0	28.8	16.7	0.8	15.0	12.0	58.3

APPENDIX 6 - Cell Separation Data

				WHOLE BLOOD					SEPARATED CELLS			
Date	Subject	No.	Sample	LY	MO	Vol.	No.	% MO	MNC	Vol.	No.	% Loss
5/5/97	DM	044	/PT	1.7	0.3	17.0	34.0	15.0	0.5	15.0	7.5	77.9
5/6/97	IT	041	/4	1.9	0.9	16.0	44.8	32.1	1.2	15.0	18.0	59.8
5/6/97	CB	042	/3	2.1	0.1	15.5	34.1	4.5	1.3	15.0	19.5	42.8
5/8/97	EK	043	/2	0.6	0.4	16.0	16.0	40.0	0.2	15.0	3.0	81.3
5/8/97	DM	044	/2	1.3	0.7	16.0	32.0	35.0	0.8	15.0	12.0	62.5
5/8/97	JR	039	/5	2.2	0.5	17.0	45.9	18.5	1.5	15.0	22.5	51.0
5/12/97	DM	044	/3	2.1	0.9	16.0	48.0	30.0	1.3	15.0	19.5	59.4
5/12/97	CB	042	/4	2.7	0.7	17.0	57.8	20.6	1.9	15.0	28.5	50.7
5/14/97	EK	043	/1R1	1.0	1.2	16.0	35.2	54.5	0.7	15.0	10.5	70.2
5/14/97	FB	045	/PT	1.2	0.3	16.0	24.0	20.0	0.9	15.0	13.5	43.8
5/16/97	FB	045	/2	0.7	0.2	16.0	14.4	22.2	0.5	15.0	7.5	47.9
5/16/97	ER	046	/PT	1.4	0.3	16.0	27.2	17.6	0.9	15.0	13.5	50.4
5/19/97	DM	044	/4	3.3	0.5	16.0	60.8	13.2	1.6	16.0	25.6	57.9
5/19/97	ER	046	/2	1.4	1.1	16.5	41.3	44.0	0.8	15.0	12.0	70.9
5/20/97	FB	045	/3	1.1	0.3	17.0	23.8	21.4	0.8	15.0	12.0	49.6
5/22/97	ER	046	/3	1.6	1.5	17.0	52.7	48.4	1.2	15.0	18.0	65.8
5/22/97	EK	043	/1R2	2.2	1.1	17.0	56.1	33.3	1.6	15.0	24.0	57.2
5/27/97	IT	041	/5	0.8	1.1	16.5	31.4	57.9	0.5	15.0	7.5	76.1
5/27/97	ER	046	/1R1	1.4	2.0	16.5	56.1	58.8	0.7	15.0	10.5	81.3
5/28/97	RM	047	/PT	2.3	0.7	17.0	51.0	23.3	1.7	15.0	25.5	50.0
5/30/97	RM	047	/2	0.5	0.5	16.5	16.5	50.0	0.4	15.0	6.0	63.6
6/2/97	FB	045	/1R1	0.2	0.6	16.0	12.8	75.0	0.1	15.0	1.5	88.3
6/2/97	RM	047	/3	1.8	0.5	16.0	36.8	21.7	1.3	15.0	19.5	47.0
6/2/97	CB	042	/5	2.4	0.4	17.0	47.6	14.3	1.0	15.0	15.0	68.5
6/2/97	DM	044	/5	0.6	0.2	14.0	11.2	25.0	0.2	15.0	3.0	73.2
6/9/97	ER	046	/2R1	1.3	0.7	16.0	32.0	35.0	0.9	15.0	13.5	57.8
6/9/97	RM	047	/4	1.9	0.7	16.0	41.6	26.9	1.8	15.0	27.0	35.1
6/9/97	FB	045	/1R2	0.4	0.6	16.0	16.0	60.0	0.3	15.0	4.5	71.9
6/16/97	EK	043	/1R3	3.0	0.7	16.0	59.2	18.9	1.1	15.0	16.5	72.1
6/16/97	ER	046	/2R2	0.8	0.7	16.0	24.0	46.7	0.6	15.0	9.0	62.5
7/7/97	FB	045	/1R3	0.4	0.2	15.5	9.3	33.3	NR	15.0	NR	NR
7/7/97	ER	046	/2R3	0.6	0.4	16.5	16.5	40.0	0.4	15.0	6.0	63.6
7/7/97	RM	047	/5	1.0	0.6	16.5	26.4	37.5	0.9	15.0	13.5	48.9

APPENDIX 6 - Cell Separation Data

	WHOLE BLOOD					SEPARATED CELLS			
	LY	MO	Vol.	No.	% MO	MNC	Vol.	No.	% Loss
Average	1.4	0.7	15.0	31.2	34.2	0.9	15.1	13.1	58.9
Median	1.3	0.6	15.5	28.8	32.8	0.8	15.0	12.0	58.2
Maximum	4.4	2.1	19.0	71.6	75.0	3.0	17.0	45.0	92.9
Minimum	0.1	0.0	8.1	1.6	0.0	0.1	15.0	1.5	6.3
5th Percentile	0.3	0.2	13.0	10.8	12.5	0.2	15.0	3.0	37.5
95th Percentile	2.9	1.3	17.0	57.7	60.0	2.0	16.0	29.7	79.7
1st Quartile	0.8	0.4	13.5	20.2	23.9	0.5	15.0	7.5	50.6
3rd Quartile	1.8	0.9	16.0	40.3	43.8	1.1	15.0	16.5	67.7

Notes:

LY = Lymphocyte count (in millions per ml)

MO = Monocyte count (in millions per ml)

MNC = Mononuclear cell count (in millions per ml)

Vol. = Volume of blood or buffered saline resuspending the separated cells

No. = Number of mononuclear cells in whole blood or separated cells

% Loss = Percentage of mononuclear cells lost during cell separation

NR = No results available

APPENDIX 7 - RNA Quantitation Data

Date	Delay	Subject	No.	Sample	MNC	Purity 260/280	RNA Conc. (microg/mL)	RNA Vol. (microlitres)	RNA Quantity (micrograms)	RNA Quantity Per 10^6 Cells
5/15/96	1	EP	001	CAPD, Pre-Tx	7.8	2.047	365.6	30	10.968	1.406
5/17/96	1	EP	001	Day 2	3.9	2.021	284.4	20	5.688	1.458
5/21/96	1	EP	001	Day 6	7.5	2.092	480.0	20	9.600	1.280
5/28/96	4	EP	001	Day 10	10.4	1.987	438.6	20	8.772	0.843
6/12/96	2	EP	001	Day 27	7.8	2.041	460.2	20	9.204	1.180
5/28/96	2	FO	003	Pre-dialysis, Pre-Tx	3.9	2.039	369.2	20	7.384	1.893
5/30/96	1	FO	003	Day 3	2.6	2.038	792.8	20	15.856	6.098
6/4/96	4	FO	003	Day 5	2.6	1.910	219.4	40	8.776	3.375
6/12/96	5	FO	003	Day 12	5.2	2.048	398.2	30	11.946	2.297
6/26/96	2	FO	003	Day 29	3.9	2.082	245.2	40	9.808	2.515
5/28/96	1	PH	005	CAPD, Pre-Tx	15.6	2.008	414.4	40	16.576	1.063
5/30/96	1	PH	005	Day 2	9.1	2.037	788.4	20	15.768	1.733
6/4/96	1	PH	005	Day 7	NR	1.721	462.0	40	18.480	NR
6/12/96	5	PH	005	Day 11	36.4	1.860	942.0	40	37.680	1.035
6/27/96	2	PH	005	Day 29, Pre-MP	11.7	1.949	430.4	30	12.912	1.104
7/3/96	5	PH	005	Day 32	3.9	1.823	230.0	15	3.450	0.885
7/9/96	4	PH	005	Day 39	10.4	1.823	361.8	30	10.854	1.044
5/30/96	1	BR	006	CAPD, Pre-Tx	13.0	2.078	594.4	20	11.888	0.914
6/4/96	4	BR	006	Day 2	3.9	1.879	218.8	20	4.376	1.122
6/6/96	1	BR	006	Day 7, Pre-MP	16.9	2.081	536.8	40	21.472	1.271
6/12/96	2	BR	006	Day 12	13.0	1.844	469.2	30	14.076	1.083
6/19/96	2	BR	006	Day 19, Pre-MP2	11.7	1.981	462.2	40	18.488	1.580
6/27/96	2	BR	006	Day 27	7.8	2.035	422.6	40	16.904	2.167
7/3/96	2	BR	006	Day 33	9.1	2.083	555.2	40	22.208	2.440
5/30/96	1	PG	007	HD, Pre-Tx	5.2	2.100	626.0	20	12.520	2.408

APPENDIX 7 - RNA Quantitation Data

Date	Delay	Subject	No.	Sample	MNC	Purity 260/280	RNA Conc. (microg/mL)	RNA Vol. (microlitres)	RNA Quantity (micrograms)	RNA Quantity Per 10^6 Cells
6/4/96	4	PG	007	Day 2	2.6	1.714	234.4	20	4.688	1.803
6/6/96	1	PG	007	Day 7	3.9	2.084	285.6	30	8.568	2.197
6/12/96	2	PG	007	Day 12	5.2	2.080	279.2	40	11.168	2.148
7/3/96	1	PG	007	Day 34	5.2	2.005	401.6	20	8.032	1.545
6/6/96	1	JM	008	Pre-dialysis, Pre-Tx	13.0	2.069	287.2	40	11.488	0.884
6/12/96	5	JM	008	Day 2	2.6	2.026	163.4	20	3.268	1.257
6/13/96	2	JM	008	Day 6	6.5	2.050	370.8	20	7.416	1.141
6/20/96	3	JM	008	Day 12, Pre-MP	5.2	2.076	316.6	20	6.332	1.218
6/26/96	1	JM	008	Day 20	1.3	1.972	90.8	20	1.816	1.397
7/3/96	2	JM	008	Day 26	3.9	1.895	191.2	20	3.824	0.981
6/13/96	2	JW	009	HD, Pre-Tx	7.8	2.090	379.0	30	11.370	1.458
6/19/96	5	JW	009	Day 2	3.9	1.846	232.0	20	4.640	1.190
6/20/96	2	JW	009	Day 6	11.7	2.060	342.6	40	13.704	1.171
6/26/96	2	JW	009	Day 12	13.0	1.942	455.0	40	18.200	1.400
7/16/96	1	JW	009	Day 33	6.5	2.087	327.6	40	13.104	2.016
6/13/96	2	EJ	010	CAPD, Pre-Tx	6.5	2.112	312.4	20	6.248	0.961
6/19/96	5	EJ	010	Day 3	3.9	2.094	279.8	20	5.596	1.435
6/20/96	2	EJ	010	Day 7	11.7	1.942	271.2	40	10.848	0.927
6/27/96	2	EJ	010	Day 14	10.4	2.043	391.2	30	11.736	1.128
7/3/96	1	EJ	010	Day 21, Pre-MP	5.2	1.860	231.5	20	4.630	0.890
7/18/96	1	EJ	010	Day 36	2.6	1.973	199.7	15	2.996	1.152
6/20/96	2	SK	011	HD, Pre-Tx	7.8	1.873	220.3	30	6.609	0.847
7/3/96	5	SK	011	Day 3	16.9	2.074	284.4	20	5.688	0.337
7/3/96	2	SK	011	Day 6	39.0	2.038	360.0	40	14.400	0.369
7/9/96	1	SK	011	Day 13	39.0	1.996	207.6	50	10.380	0.266

APPENDIX 7 - RNA Quantitation Data

Date	Delay	Subject	No.	Sample	MNC	Purity 260/280	RNA Conc. (microg/mL)	RNA Vol. (microlitres)	RNA Quantity (micrograms)	RNA Quantity Per 10^6 Cells
7/24/96	2	SK	011	Day 27	29.9	2.033	754.4	40	30.176	1.009
7/9/96	1	DR	013	HD, Pre-Tx	9.1	2.090	341.4	30	10.242	1.125
7/16/96	4	DR	013	Day 3	6.5	2.069	345.6	20	6.912	1.063
7/16/96	1	DR	013	Day 6	13.0	2.094	636.0	40	25.440	1.957
7/24/96	1	DR	013	Day 14	7.8	2.089	532.0	40	21.280	2.728
8/6/96	1	DR	013	Day 27	9.1	2.060	379.0	40	15.160	1.666
7/11/96	1	TG	014	HD, Pre-Tx	23.4	2.076	669.2	40	26.768	1.144
7/16/96	4	TG	014	Day 2	5.2	2.087	315.2	20	6.304	1.212
7/18/96	1	TG	014	Day 7, Pre-MP	11.7	2.112	508.8	30	15.264	1.305
7/24/96	2	TG	014	Day 12	13.0	2.001	443.8	40	17.752	1.366
7/30/96	1	TG	014	Day 19	7.8	2.088	315.6	40	12.624	1.618
7/11/96	1	MP	015	CAPD, Pre-Tx	9.1	2.071	328.4	40	13.136	1.444
7/16/96	4	MP	015	Day 2	10.4	2.084	328.8	40	13.152	1.265
7/18/96	1	MP	015	Day 7	15.6	2.087	584.4	40	23.376	1.498
7/24/96	2	MP	015	Day 12	24.7	2.075	1101.0	40	44.040	1.783
8/6/96	1	MP	015	Day 26, Post-MP	14.3	2.114	533.2	40	21.328	1.491
9/2/96	17	MP	015	Day 37	2.9	1.960	93.9	20	1.878	0.648
10/8/96	6	BT	016	Pre-dialysis, Pre-Tx	11.6	2.086	290.2	30	8.706	0.751
10/8/96	4	BT	016	Day 2	NR	2.087	263.2	30	7.896	NR
10/8/96	1	BT	016	Day 5	7.3	2.007	408.0	30	12.240	1.688
10/16/96	2	BT	016	Day 12	11.6	1.898	471.2	40	18.848	1.625
11/13/96	2	BT	016	Day 40	13.1	2.031	699.9	40	27.996	2.145
10/8/96	1	JS	017	Pre-dialysis, Pre-Tx	18.9	2.091	469.2	40	18.768	0.996
10/16/96	5	JS	017	Day 2	NR	2.042	393.6	40	15.744	NR
10/16/96	2	JS	017	Day 5	14.5	1.883	461.8	40	18.472	1.274

APPENDIX 7 - RNA Quantitation Data

Date	Delay	Subject	No.	Sample	MNC	Purity 260/280	RNA Conc. (microg/mL)	RNA Vol. (microlitres)	RNA Quantity (micrograms)	RNA Quantity Per 10^6 Cells
10/22/96	1	JS	017	Day 12	42.1	1.828	934.4	60	56.064	1.333
11/13/96	2	JS	017	Day 33	30.5	2.032	1159.8	60	69.588	2.285
10/16/96	1	PC	018	HD, Pre-Tx	14.5	1.986	464.6	40	18.584	1.282
10/22/96	4	PC	018	Day 3	13.1	2.090	294.6	40	11.784	0.903
10/22/96	1	PC	018	Day 6	13.1	2.104	568.4	40	22.736	1.742
10/30/96	2	PC	018	Day 13	21.8	1.938	666.6	40	26.664	1.226
11/13/96	2	PC	018	Day 27	11.6	2.076	480.4	40	19.216	1.657
10/22/96	1	EC	019	Pre-dialysis, Pre-Tx	21.8	2.060	566.7	40	22.668	1.042
10/30/96	5	EC	019	Day 2	4.4	2.080	324.8	20	6.496	1.493
10/30/96	2	EC	019	Day 5, Pre-MP	8.7	2.079	459.3	30	13.779	1.584
11/5/96	4	EC	019	Day 9	10.9	2.054	790.8	20	15.816	1.458
11/13/96	5	EC	019	Day 16	16.0	2.075	304.6	40	12.184	0.764
11/27/96	8	EC	019	Day 27	14.5	2.071	582.0	20	11.640	0.803
10/30/96	1	RF	020	HD, Pre-Tx	8.7	2.092	555.2	30	16.656	1.914
11/5/96	4	RF	020	Day 3	12.4	2.079	332.4	40	13.296	1.072
11/5/96	1	RF	020	Day 6	16.0	1.970	639.3	40	25.572	1.603
11/21/96	10	RF	020	Day 13	18.9	2.049	574.2	60	34.452	1.828
11/27/96	2	RF	020	Day 27	31.9	1.878	694.8	60	41.688	1.307
10/30/96	1	AB	021	Pre-dialysis, Pre-Tx	5.8	2.043	399.6	20	7.992	1.378
11/5/96	4	AB	021	Day 3	4.4	2.034	380.2	20	7.604	1.748
11/5/96	1	AB	021	Day 6	5.8	2.085	519.2	20	10.384	1.790
11/21/96	10	AB	021	Day 13	1.5	1.956	165.8	15	2.487	1.715
11/27/96	8	AB	021	Day 21, Pre-MP	5.8	2.065	276.2	20	5.524	0.952
11/27/96	5	AB	021	Day 24	2.9	2.001	219.1	15	3.287	1.133
12/3/96	1	AB	021	Day 34	11.6	1.987	437.2	40	17.488	1.508

APPENDIX 7 - RNA Quantitation Data

Date	Delay	Subject	No.	Sample	MNC	Purity 260/280	RNA Conc. (microg/mL)	RNA Vol. (microlitres)	RNA Quantity (micrograms)	RNA Quantity Per 10^6 Cells
12/20/96	1	AB	021	Day 51	11.6	2.034	333.9	50	16.695	1.439
11/13/96	7	RE	022	HD, Pre-Tx	11.6	1.956	416.6	20	8.332	0.718
11/13/96	5	RE	022	Day 2	11.6	2.042	342.0	40	13.680	1.179
11/21/96	9	RE	022	Day 6	26.1	2.066	498.6	60	29.916	1.146
11/26/96	8	RE	022	Day 12	17.4	2.077	501.6	40	20.064	1.153
12/10/96	1	RE	022	Day 33	4.4	2.083	311.4	20	6.228	1.432
11/21/96	9	NH	023	HD, Pre-Tx	11.6	1.994	422.4	30	12.672	1.092
11/26/96	11	NH	023	Day 3	10.2	1.967	423.6	20	8.472	0.835
11/26/96	8	NH	023	Day 6	13.1	1.970	433.2	20	8.664	0.664
11/27/96	5	NH	023	Day 10, Pre-MP	14.5	1.861	451.4	40	18.056	1.245
12/3/96	8	NH	023	Day 13, Pre-MP2	5.8	2.064	271.2	20	5.424	0.935
12/3/96	1	NH	023	Day 20	5.8	2.073	301.2	30	9.036	1.558
12/10/96	1	NH	023	Day 27	2.9	2.012	406.2	20	8.124	2.801
1/2/97	10	NH	023	Day 41	8.7	1.968	216.6	30	6.498	0.747
11/21/96	9	BM	024	CAPD, Pre-Tx	21.8	2.113	401.4	60	24.084	1.107
11/26/96	11	BM	024	Day 3	20.3	2.081	314.8	40	12.592	0.620
11/26/96	8	BM	024	Day 6	16.0	1.899	414.2	20	8.284	0.519
12/3/96	8	BM	024	Day 13	14.5	2.030	394.0	30	11.820	0.815
12/10/96	1	BM	024	Day 27	10.2	2.035	365.2	20	7.304	0.720
11/21/96	7	VH	025	CAPD, Pre-Tx	8.7	2.070	262.6	30	7.878	0.906
11/26/96	8	VH	025	Day 4	7.3	2.067	171.8	20	3.436	0.474
11/27/96	7	VH	025	Day 6, Pre-MP	7.3	2.078	245.8	20	4.916	0.678
12/3/96	8	VH	025	Day 11	10.2	2.063	317.0	30	9.510	0.937
12/3/96	1	VH	025	Day 18	13.1	1.961	425.8	40	17.032	1.305
12/20/96	3	VH	025	Day 33	11.6	2.064	455.4	30	13.662	1.178

APPENDIX 7 - RNA Quantitation Data

Date	Delay	Subject	No.	Sample	MNC	Purity 260/280	RNA Conc. (microg/mL)	RNA Vol. (microlitres)	RNA Quantity (micrograms)	RNA Quantity Per 10^6 Cells
12/16/96	5	SM	026	HD, Pre-Tx	11.6	1.911	450.8	20	9.016	0.777
12/16/96	3	SM	026	Day 2	NR	2.087	278.0	20	5.560	NR
12/20/96	3	SM	026	Day 6	20.3	1.942	451.4	30	13.542	0.667
1/2/97	10	SM	026	Day 12	18.9	2.061	231.4	30	6.942	0.368
1/14/97	1	SM	026	Day 33	20.3	2.067	559.2	30	16.776	0.826
1/27/97	5	KB	027	Pre-dialysis, Pre-Tx	21.8	2.001	454.0	40	18.160	0.835
1/27/97	3	KB	027	Day 2	7.3	2.071	257.8	20	5.156	0.711
2/6/97	8	KB	027	Day 7	30.5	2.074	802.8	30	24.084	0.791
2/6/97	3	KB	027	Day 12	21.8	1.963	641.7	50	32.085	1.475
3/12/97	6	KB	027	Day 43	10.2	2.063	438.0	40	17.520	1.726
2/6/97	3	JH	029	HD, Pre-Tx	20.3	1.956	439.2	30	13.176	0.649
2/6/97	1	JH	029	Day 2	8.7	2.052	353.0	30	10.590	1.217
2/13/97	3	JH	029	Day 7	18.9	2.030	354.2	60	21.252	1.127
2/18/97	4	JH	029	Day 11, Pre-MP	18.9	2.000	303.6	30	9.108	0.483
2/20/97	1	JH	029	Day 16, Pre-ATG	11.6	2.072	491.1	40	19.644	1.693
3/12/97	7	JH	029	Day 30	1.5	2.008	96.9	15	1.454	1.002
3/14/97	1	JH	029	Day 38	10.2	1.924	829.2	50	41.460	4.085
4/8/97	1	JH	029	Day 63	7.3	1.825	436.0	30	13.080	1.804
2/6/97	2	CA	030	HD, Pre-Tx	20.3	2.062	370.8	40	14.832	0.731
2/13/97	6	CA	030	Day 3	13.1	2.021	275.0	30	8.250	0.632
2/18/97	7	CA	030	Day 7	26.1	1.965	356.6	50	17.830	0.683
2/18/97	1	CA	030	Day 13	30.5	2.024	840.0	50	42.000	1.379
3/4/97	1	CA	030	Day 27	8.7	2.055	708.4	20	14.168	1.629
2/6/97	2	RW	031	HD, Pre-Tx	11.6	2.061	522.4	30	15.672	1.351
2/13/97	6	RW	031	Day 3	4.4	1.900	100.6	30	3.018	0.694

APPENDIX 7 - RNA Quantitation Data

Date	Delay	Subject	No.	Sample	MNC	Purity 260/280	RNA Conc. (microg/mL)	RNA Vol. (microlitres)	RNA Quantity (micrograms)	RNA Quantity Per 10^6 Cells
2/13/97	2	RW	031	Day 7	14.5	2.070	297.4	60	17.844	1.231
2/18/97	1	RW	031	Day 13	8.7	2.000	684.4	30	20.532	2.360
3/4/97	1	RW	031	Day 27	4.4	2.045	573.0	20	11.460	2.634
2/13/97	8	GK	032	CAPD, Pre-Tx	14.5	2.067	235.8	30	7.074	0.488
2/13/97	6	GK	032	Day 2	20.3	2.046	353.6	30	10.608	0.523
2/18/97	6	GK	032	Day 7	34.8	2.009	857.4	30	25.722	0.739
2/18/97	1	GK	032	Day 12	34.8	1.969	751.6	60	45.096	1.296
3/12/97	6	GK	032	Day 29	20.3	2.090	378.6	40	15.144	0.746
2/13/97	3	AA	033	HD, Pre-Tx	7.3	1.972	224.6	30	6.738	0.929
2/18/97	6	AA	033	Day 2	5.8	1.974	183.6	15	2.754	0.475
2/20/97	3	AA	033	Day 7	13.1	2.040	763.2	20	15.264	1.170
2/25/97	4	AA	033	Day 11	17.4	2.048	476.4	15	7.146	0.411
3/12/97	2	AA	033	Day 28	13.1	2.077	357.0	40	14.280	1.094
2/20/97	1	NR	034	HD, Pre-Tx	15.5	2.073	493.8	40	19.752	1.274
2/25/97	4	NR	034	Day 2, Pre-MP/ATG	11.6	2.039	791.6	15	11.874	1.024
3/12/97	5	NR	034	Day 16	2.9	2.010	176.4	20	3.528	1.217
3/18/97	1	NR	034	Day 26	1.5	1.594	70.6	10	0.706	0.487
4/8/97	1	NR	034	Day 47	1.5	2.002	108.0	10	1.080	0.745
2/25/97	4	LH	035	HD, Pre-Tx	5.8	2.053	335.6	20	6.712	1.157
2/25/97	1	LH	035	Day 2	4.4	2.011	599.4	10	5.994	1.378
2/28/97	1	LH	035	Day 5	2.9	2.032	357.0	10	3.570	1.231
3/12/97	5	LH	035	Day 13	5.8	2.054	203.8	20	4.076	0.703
3/25/97	1	LH	035	Day 30	4.4	1.964	224.3	20	4.486	1.031
2/28/97	4	DK	036	HD, Pre-Tx	10.2	1.824	442.8	15	6.642	0.654
2/28/97	1	DK	036	Day 3	14.5	2.068	449.7	40	17.988	1.241

APPENDIX 7 - RNA Quantitation Data

Date	Delay	Subject	No.	Sample	MNC	Purity 260/280	RNA Conc. (microg/mL)	RNA Vol. (microlitres)	RNA Quantity (micrograms)	RNA Quantity Per 10^6 Cells
3/4/97	1	DK	036	Day 7	17.4	2.074	668.0	40	26.720	1.536
3/14/97	4	DK	036	Day 14	24.7	1.923	796.8	30	23.904	0.970
3/25/97	1	DK	036	Day 28	17.4	2.090	546.8	40	21.872	1.257
2/28/97	4	KB	037	CAPD, Pre-Tx	10.2	2.078	692.1	10	6.921	0.682
2/28/97	1	KB	037	Day 3	8.7	2.036	568.8	20	11.376	1.308
3/4/97	1	KB	037	Day 7	17.4	2.028	614.1	40	24.564	1.412
3/14/97	4	KB	037	Day 14	16.0	1.930	528.3	30	15.849	0.994
3/25/97	1	KB	037	Day 28	8.7	2.084	328.8	20	6.576	0.756
3/12/97	7	IL	038	Pre-dialysis, Pre-Tx	13.1	2.048	276.0	20	5.520	0.423
3/12/97	5	IL	038	Day 2	2.9	2.020	167.8	15	2.517	0.868
3/14/97	1	IL	038	Day 8	5.8	1.989	765.0	50	38.250	6.595
3/18/97	1	IL	038	Day 12	7.3	2.066	574.5	60	34.470	4.754
4/8/97	1	IL	038	Day 33	20.3	2.074	543.6	50	27.180	1.339
3/25/97	6	JR	039	HD, Pre-Tx	14.5	2.080	275.2	30	8.256	0.569
4/8/97	4	JR	039	Day 2	11.6	2.122	218.1	10	2.181	0.188
4/16/97	7	JR	039	Day 7	30.5	1.943	427.2	60	25.632	0.842
4/16/97	2	JR	039	Day 12	26.1	2.051	714.4	60	42.864	1.642
5/9/97	1	JR	039	Day 36	21.8	2.042	486.3	60	29.178	1.342
3/25/97	4	MS	040	Pre-dialysis, Pre-Tx	11.6	2.016	213.4	15	3.201	0.276
3/25/97	1	MS	040	Day 3	10.2	2.084	314.7	40	12.588	1.240
4/1/97	5	MS	040	Day 6	14.5	2.004	205.1	40	8.204	0.566
4/8/97	4	MS	040	Day 14	10.2	2.035	153.4	20	3.068	0.302
4/22/97	1	MS	040	Day 31	4.4	2.090	298.2	15	4.473	1.028
5/1/97	7	IT	041	CAPD, Pre-Tx	21.8	2.012	322.6	50	16.130	0.742
5/1/97	3	IT	041	Day 4	7.3	2.033	171.3	30	5.139	0.709

APPENDIX 7 - RNA Quantitation Data

Date	Delay	Subject	No.	Sample	MNC	Purity 260/280	RNA Conc. (microg/mL)	RNA Vol. (microlitres)	RNA Quantity (micrograms)	RNA Quantity Per 10^6 Cells
5/1/97	1	IT	041	Day 6	11.6	2.071	340.0	50	17.000	1.466
5/7/97	1	IT	041	Day 12	17.4	2.077	622.0	40	24.880	1.430
5/29/97	2	IT	041	Day 33	7.3	2.057	356.0	20	7.120	0.982
5/1/97	1	CB	042	HD, Pre-Tx	14.5	2.043	393.0	40	15.720	1.084
5/7/97	5	CB	042	Day 2	10.2	2.059	122.2	20	2.444	0.241
5/7/97	1	CB	042	Day 6	18.9	2.060	579.6	40	23.184	1.230
5/21/97	9	CB	042	Day 12	27.6	2.050	371.6	30	11.148	0.405
6/3/97	1	CB	042	Day 33	14.5	2.070	419.7	60	25.182	1.737
5/7/97	2	EK	043	HD, Pre-Tx	11.6	2.062	318.8	20	6.376	0.550
5/9/97	1	EK	043	Day 3, Pre-MP	2.9	2.029	186.2	20	3.724	1.284
5/21/97	7	EK	043	Day 9	10.2	2.039	207.8	20	4.156	0.409
5/23/97	1	EK	043	Day 17	23.2	2.031	594.6	80	47.568	2.050
6/18/97	2	EK	043	Day 42	16.0	2.082	708.8	60	42.528	2.666
5/7/97	2	DM	044	Pre-dialysis, Pre-Tx	7.3	2.059	157.1	15	2.357	0.325
5/9/97	1	DM	044	Day 3	11.6	2.037	354.8	40	14.192	1.223
5/21/97	9	DM	044	Day 7	18.9	2.063	273.0	30	8.190	0.434
5/23/97	4	DM	044	Day 14	24.8	2.030	385.2	60	23.112	0.932
6/3/97	1	DM	044	Day 28	2.9	2.091	265.0	20	5.300	1.828
5/21/97	7	FB	045	HD, Pre-Tx	13.1	2.057	237.0	20	4.740	0.363
5/21/97	5	FB	045	Day 2	7.3	2.053	287.4	15	4.311	0.595
5/23/97	3	FB	045	Day 6, Pre-MP/ATG	11.6	1.966	432.4	30	12.972	1.118
6/3/97	1	FB	045	Day 19	1.5	2.022	74.3	20	1.486	1.025
6/18/97	9	FB	045	Day 26	4.4	2.053	200.6	20	4.012	0.922
7/8/97	1	FB	045	Day 54	NR	1.965	230.7	20	4.614	NR
5/21/97	5	ER	046	Pre-dialysis, Pre-Tx	13.1	2.073	298.6	20	5.972	0.458

APPENDIX 7 - RNA Quantitation Data

Date	Delay	Subject	No.	Sample	MNC	Purity 260/280	RNA Conc. (microg/mL)	RNA Vol. (microlitres)	RNA Quantity (micrograms)	RNA Quantity Per 10^6 Cells
5/23/97	4	ER	046	Day 3	11.6	2.043	373.6	30	11.208	0.966
5/23/97	1	ER	046	Day 6, Pre-MP	17.4	2.079	402.2	60	24.132	1.387
5/29/97	2	ER	046	Day 11, Pre-OKT3	10.2	2.087	475.6	20	9.512	0.937
6/18/97	9	ER	046	Day 24	13.1	2.069	263.4	20	5.268	0.404
6/18/97	2	ER	046	Day 31	8.7	2.078	266.6	40	10.664	1.226
7/8/97	1	ER	046	Day 52	5.8	2.094	289.2	30	8.676	1.496
5/29/97	1	RM	047	HD, Pre-Tx	24.7	2.074	928.2	40	37.128	1.506
6/3/97	4	RM	047	Day 2	5.8	2.082	212.6	20	4.252	0.733
6/3/97	1	RM	047	Day 5	18.9	2.066	578.8	60	34.728	1.842
6/18/97	9	RM	047	Day 12	26.1	2.008	410.0	60	24.600	0.943
7/8/97	1	RM	047	Day 40	13.1	2.048	391.2	50	19.560	1.499
Average:	3				**12.4**	**2.0**	**413.3**	**32.0**	**14.2**	**1.257**
Median:	2				**11.6**	**2.0**	**379.6**	**30.0**	**11.8**	**1.153**
Maximum:	17				**42.1**	**2.1**	**1159.8**	**80.0**	**69.6**	**6.595**
Minimum:	1				**1.3**	**1.6**	**70.6**	**10.0**	**0.7**	**0.188**
1st Quartile:	1				**6.5**	**2.0**	**278.9**	**20**	**6.6**	**0.821**
3rd Quartile:	5				**16.0**	**2.1**	**503.4**	**40**	**18.5**	**1.483**
5th %tile:	1				**2.9**	**1.9**	**167.3**	**15.0**	**2.9**	**0.407**
95th %tile:	9				**28.7**	**2.1**	**791.0**	**60.0**	**37.3**	**2.384**

Notes:

Delay = No. of days between cell separation & RNA extraction

MNC = Number of mononuclear cells

Vol. = Volume

Conc. = Concentration

%tile = Percentiles

APPENDIX 8

PCR ELISA Data (Non-Rejectors)

Patients	Sample	IL-2	IL-2 %C	IL-4	IL-4 %C	IFN-G	IFN-G %C	IL-10	IL-10 %C
PC018	/PT	0.80	100.00	1.29	100.00	1.90	100.00	0.49	100.00
PC018	/2	0.44	70.36	1.04	77.53	1.92	101.41	0.48	99.00
PC018	/3	0.25	57.81	0.97	72.54	1.53	68.73	0.68	120.50
PC018	/4	0.27	59.07	1.09	81.46	1.52	68.56	0.70	122.69
PC018	/5	0.57	79.57	0.44	42.66	1.16	47.64	0.02	62.69
JS017	/PT	2.06	100.00	2.20	100.00	2.37	100.00	1.71	100.00
JS017	/2	0.74	26.63	1.37	43.87	2.39	101.46	1.70	98.56
JS017	/3	0.64	24.20	1.78	65.51	2.08	74.90	1.59	88.25
JS017	/4	0.28	16.86	1.69	59.96	1.29	33.74	1.11	54.88
JS017	/5	0.24	16.17	1.19	36.31	1.81	57.04	1.02	50.21
CA030	/PT	0.07	100.00	1.61	100.00	1.47	100.00	0.07	100.00
CA030	/2	0.18	112.02	1.62	101.06	1.86	147.18	0.27	122.63
CA030	/3	0.47	148.51	1.77	118.29	2.16	199.77	0.93	236.32
CA030	/4	0.08	101.21	1.77	118.29	2.10	187.95	1.24	321.23
CA030	/5	0.08	100.90	1.42	82.86	1.92	156.36	0.44	144.27
KB027	/PT	0.09	100.00	1.38	100.00	2.10	100.00	0.08	100.00
KB027	/2	0.10	101.26	0.68	49.93	2.17	107.09	0.96	239.05
KB027	/3	0.13	104.50	0.87	59.99	1.72	68.59	0.16	108.06
KB027	/4	0.12	102.84	0.93	63.83	1.35	47.35	0.08	100.00
KB027	/5	0.25	118.12	1.76	146.52	1.58	59.51	0.10	101.51
SM026	/PT	0.62	100.00	1.34	100.00	2.22	100.00	0.07	100.00
SM026	/2	0.43	82.86	0.17	31.08	2.09	87.94	0.72	190.50
SM026	/3	0.78	117.53	0.92	65.54	1.97	77.84	0.36	132.84
SM026	/4	0.41	81.38	1.44	110.79	2.31	109.91	0.03	96.08
SM026	/5	0.17	64.34	1.16	82.99	1.74	61.88	0.18	111.74
RE022	/PT	0.05	100.00	0.77	100.00	1.93	100.00	0.09	100.00
RE022	/2	1.54	446.60	0.00	46.46	1.74	82.20	0.88	221.22
RE022	/3	0.10	105.28	0.18	55.49	2.17	127.12	0.29	122.14
RE022	/4	0.13	109.14	0.15	53.71	1.35	56.16	1.04	258.18
RE022	/5	0.57	168.96	1.39	185.99	1.56	68.90	1.83	566.89
BM024	/PT	0.16	100.00	0.88	100.00	2.12	100.00	0.44	100.00
BM024	/2	0.80	190.60	0.28	55.02	2.16	103.30	0.38	94.46
BM024	/3	0.23	107.73	1.29	150.53	1.67	63.41	1.20	214.04
BM024	/4	0.42	129.95	1.73	235.26	2.11	98.86	0.14	74.01
BM024	/5	1.29	311.58	1.69	226.03	2.30	119.48	1.61	324.79
RF020	/PT	0.95	100.00	2.15	100.00	1.89	100.00	1.90	100.00
RF020	/2	0.44	59.96	1.16	36.97	1.72	85.00	1.90	99.60
RF020	/3	0.48	62.88	1.94	80.90	1.34	57.75	1.57	71.89
RF020	/4	0.25	49.68	1.24	40.31	1.24	52.23	0.90	36.66
RF020	/5	0.13	43.96	0.90	28.64	0.43	23.34	0.14	17.08
BT016	/PT	1.23	100.00	1.74	100.00	2.31	100.00	0.87	100.00
BT016	/2	0.28	38.79	0.10	19.48	2.06	78.31	1.79	250.68
BT016	/3	0.31	39.83	0.86	41.60	1.93	68.35	1.63	214.58
BT016	/4	0.52	48.94	1.92	120.26	1.97	71.68	1.82	258.31
BT016	/5	0.41	44.13	1.80	106.61	2.10	80.98	1.73	237.26

APPENDIX 8

PCR ELISA Data (Non-Rejectors)

Patients	Sample	IL-2	IL-2 %C	IL-4	IL-4 %C	IFN-G	IFN-G %C	IL-10	IL-10 %C
EP001	/PT	0.60	100.00	1.95	100.00	2.05	100.00	1.33	100.00
EP001	/2	0.27	71.78	1.51	63.92	1.67	67.98	1.14	82.82
EP001	/3	0.54	93.52	1.80	85.98	1.69	69.38	1.91	177.71
EP001	/4	0.88	132.05	1.63	72.51	2.32	130.08	0.29	35.24
EP001	/5	0.72	112.69	0.82	32.14	1.92	87.72	1.76	153.04
JW009	/PT	0.36	100.00	0.72	100.00	0.67	100.00	1.31	100.00
JW009	/2	0.59	126.05	0.32	66.63	1.59	250.18	0.93	68.18
JW009	/3	0.51	116.24	0.27	63.44	1.46	220.78	1.52	123.86
JW009	/4	0.45	109.64	0.83	111.57	1.19	168.54	1.61	135.19
JW009	/5	0.45	109.86	0.93	122.57	1.84	323.17	1.98	196.01
SK011	/PT	0.42	100.00	1.04	100.00	0.85	100.00	0.90	100.00
SK011	/2	0.61	120.74	0.25	45.41	1.90	284.34	1.13	126.81
SK011	/3	0.67	128.79	1.10	106.77	1.64	220.23	1.55	192.51
SK011	/4	0.45	102.84	1.18	115.66	1.29	154.81	1.41	166.28
SK011	/5	0.11	73.45	1.23	121.11	1.07	124.67	2.30	404.71
DR013	/PT	0.14	100.00	0.90	100.00	1.49	100.00	0.39	100.00
DR013	/2	0.14	100.35	1.11	123.24	1.72	125.04	2.27	653.39
DR013	/3	0.06	96.46	1.13	124.92	1.14	70.26	1.80	410.21
DR013	/4	0.08	96.46	0.95	104.81	1.54	104.86	0.76	144.05
DR013	/5	0.27	114.74	1.48	177.98	1.49	99.45	2.15	579.79
PG007	/PT	0.46	100.00	1.41	100.00	1.65	100.00	0.69	100.00
PG007	/2	0.26	81.67	0.12	27.57	0.71	39.10	1.44	212.55
PG007	/3	0.90	155.82	2.03	185.80	1.56	91.58	2.02	379.43
PG007	/4	0.79	139.17	1.87	158.41	2.09	155.19	0.62	93.61
PG007	/5	0.94	161.69	2.15	209.17	2.02	144.41	2.04	387.68
FO003	/PT	0.13	100.00	1.17	100.00	0.88	100.00	1.56	100.00
FO003	/2	0.09	96.95	0.01	31.40	0.39	61.11	1.57	101.66
FO003	/3	0.15	101.46	0.01	31.44	0.56	72.61	2.00	156.05
FO003	/4	0.16	103.30	0.39	45.96	0.84	96.13	1.58	102.63
FO003	/5	0.19	106.08	0.88	75.01	0.70	83.40	1.76	123.18
RW031	/PT	0.17	100.00	1.03	100.00	1.95	100.00	0.16	100.00
RW031	/2	0.21	103.51	0.23	44.80	2.01	106.66	1.15	268.99
RW031	/3	0.04	93.19	0.19	43.13	1.05	40.45	0.09	93.15
RW031	/4	0.27	110.08	0.70	71.43	1.98	102.94	1.09	254.21
RW031	/5	0.12	95.46	1.62	179.41	2.05	110.52	1.13	264.59
GK032	/PT	0.39	100.00	1.85	100.00	2.23	100.00	0.15	100.00
GK032	/2	0.32	93.38	1.06	45.50	1.69	58.33	0.70	173.33
GK032	/3	0.25	87.50	1.76	91.81	2.39	118.41	1.01	237.26
GK032	/4	0.03	75.20	1.57	76.00	1.89	71.57	1.31	319.31
GK032	/5	1.11	205.96	1.58	76.76	2.07	85.34	0.66	167.36
KB037	/PT	0.09	100.00	1.99	100.00	1.81	100.00	0.87	100.00
KB037	/2	0.05	100.00	1.07	39.83	1.24	56.98	0.68	82.70
KB037	/3	0.04	100.00	1.01	37.53	0.38	24.06	0.21	51.38
KB037	/4	0.50	149.18	2.00	101.51	2.32	166.70	0.83	95.93
KB037	/5	0.32	124.79	1.62	69.07	2.32	167.62	1.21	140.49

APPENDIX 8

PCR ELISA Data (Non-Rejectors)

Patients	Sample	IL-2	IL-2 %C	IL-4	IL-4 %C	IFN-G	IFN-G %C	IL-10	IL-10 %C
AA033	/PT	0.14	100.00	1.45	100.00	1.66	100.00	0.10	100.00
AA033	/2	0.17	103.25	0.58	41.67	1.74	108.60	0.62	167.95
AA033	/3	0.04	95.89	0.73	48.46	2.00	141.48	0.78	196.70
AA033	/4	1.60	429.95	1.76	136.55	2.28	186.92	0.57	160.08
AA033	/5	0.20	106.18	1.01	64.56	1.87	123.61	0.20	109.64
DK036	/PT	0.41	100.00	1.78	100.00	2.08	100.00	0.37	100.00
DK036	/2	0.05	73.27	0.86	39.99	0.91	31.08	0.26	89.14
DK036	/3	0.01	73.27	0.14	19.43	0.31	17.15	0.02	70.47
DK036	/4	0.34	93.10	1.10	50.48	2.27	121.84	0.34	96.42
DK036	/5	0.09	73.27	0.50	27.72	1.85	79.89	0.30	92.96
LH035	/PT	0.01	100.00	0.58	100.00	0.33	100.00	0.02	100.00
LH035	/2	0.02	100.00	1.32	209.38	2.00	530.69	2.06	766.76
LH035	/3	0.03	100.00	0.99	149.71	2.21	658.96	0.82	222.44
LH035	/4	0.03	100.00	1.86	359.66	1.79	431.89	1.43	408.57
LH035	/5	0.13	102.84	1.62	283.20	2.00	533.35	1.57	473.51
IL038	/PT	0.23	100.00	0.28	100.00	2.46	100.00	0.28	100.00
IL038	/2	0.01	88.12	0.02	76.76	2.26	81.55	1.23	257.54
IL038	/3	0.00	88.12	0.00	75.43	1.36	33.25	0.03	77.65
IL038	/4	0.01	88.12	0.54	128.79	2.10	69.70	0.07	80.98
IL038	/5	0.02	88.12	1.56	358.59	1.32	31.85	0.27	98.41
JR039	/PT	0.11	100.00	1.25	100.00	1.93	100.00	0.16	100.00
JR039	/2	0.94	229.10	0.57	50.69	2.02	109.58	0.86	199.57
JR039	/3	0.06	99.35	0.73	59.87	1.24	50.13	0.09	92.68
JR039	/4	0.01	99.35	0.83	66.10	1.29	52.73	0.25	108.98
JR039	/5	0.07	99.35	0.96	75.31	1.11	44.13	0.21	104.86
MS040	/PT	1.28	100.00	1.86	100.00	2.17	100.00	0.23	100.00
MS040	/2	0.32	38.29	1.00	42.34	0.72	23.49	0.63	148.96
MS040	/3	0.39	41.02	1.23	53.47	1.76	66.23	0.58	141.55
MS040	/4	0.56	48.55	1.71	86.37	0.97	30.21	0.99	213.93
MS040	/5	0.31	37.66	1.71	86.55	0.75	24.29	1.55	372.48
IT041	/PT	0.32	100.00	1.15	100.00	2.06	100.00	0.11	100.00
IT041	/2	0.15	84.70	1.79	190.79	2.12	106.45	1.53	415.99
IT041	/3	0.04	80.37	0.50	52.07	1.46	55.05	0.04	93.99
IT041	/4	0.10	80.73	1.03	89.05	1.12	39.16	0.18	107.47
IT041	/5	0.10	80.57	1.51	143.40	1.87	82.61	1.12	276.21
CB042	/PT	0.02	100.00	0.53	100.00	1.02	100.00	0.15	100.00
CB042	/2	0.13	102.69	0.36	84.20	1.34	137.92	0.15	100.15
CB042	/3	0.03	100.00	0.44	91.12	0.36	51.94	0.28	114.34
CB042	/4	0.19	109.91	0.56	103.61	1.13	111.52	0.41	130.08
CB042	/5	0.03	100.00	0.39	86.94	0.62	67.13	0.16	101.51
DM044	/PT	1.09	100.00	1.15	100.00	1.95	100.00	1.39	100.00
DM044	/2	0.42	51.35	1.13	98.22	1.27	50.48	1.08	73.16
DM044	/3	0.54	57.67	1.08	92.96	1.39	56.84	0.55	43.11
DM044	/4	0.22	42.02	1.26	111.35	0.59	25.46	0.14	28.69
DM044	/5	0.23	42.61	1.13	97.78	2.02	107.14	0.56	43.87

APPENDIX 8

PCR ELISA Data (Non-Rejectors)

Patients	Sample	IL-2	IL-2 %C	IL-4	IL-4 %C	IFN-G	IFN-G %C	IL-10	IL-10 %C
RM047	/PT	0.08	100.00	1.01	100.00	1.50	100.00	0.22	100.00
RM047	/2	0.08	100.00	0.01	36.71	1.78	132.11	1.44	338.55
RM047	/3	0.01	100.00	0.00	36.53	0.22	27.83	0.03	82.78
RM047	/4	0.39	133.44	0.61	67.20	1.66	117.53	0.28	106.61
RM047	/5	0.05	100.00	1.05	104.39	2.11	183.40	0.65	154.34

APPENDIX 8

PCR ELISA Data (Non-Rejectors)

Patients	Sample	IL-5	IL-5 %C	IL-13	IL-13 %C	GrB	GrB %C	FasL	FasL %C
AA033	/PT	0.60	100.00	0.38	100.00	1.66	100.00	0.22	100.00
AA033	/2	0.75	116.47	0.28	90.12	1.70	104.55	0.31	110.41
AA033	/3	0.27	72.36	0.16	80.05	1.49	84.32	0.38	118.29
AA033	/4	1.30	202.59	0.24	86.81	1.78	112.86	0.34	113.48
AA033	/5	0.32	76.11	0.36	98.22	1.69	103.87	0.40	119.66
LH035	/PT	0.01	100.00	0.00	100.00	0.93	100.00	0.04	100.00
LH035	/2	0.04	103.46	0.06	105.92	1.62	200.27	0.32	133.11
LH035	/3	0.14	113.83	0.02	101.41	1.72	221.67	0.23	121.17
LH035	/4	0.03	101.87	0.76	212.55	1.19	130.73	0.30	129.43
LH035	/5	0.03	102.28	0.40	149.26	1.41	162.42	0.34	135.59
DK036	/PT	1.99	100.00	0.18	100.00	2.06	100.00	0.51	100.00
DK036	/2	0.18	16.36	0.09	91.67	1.10	38.29	0.29	79.97
DK036	/3	0.04	14.28	0.00	0.00	0.10	14.13	0.03	61.72
DK036	/4	0.76	29.13	0.16	98.22	2.04	98.12	0.49	97.43
DK036	/5	0.26	17.68	0.07	89.09	1.48	56.21	0.32	82.49
MS040	/PT	0.22	100.00	0.58	100.00	1.58	100.00	0.47	100.00
MS040	/2	0.10	88.74	0.26	72.69	1.46	88.69	0.44	96.85
MS040	/3	0.06	85.73	0.58	99.85	1.63	105.23	0.33	86.33
MS040	/4	0.12	90.98	0.63	104.92	1.79	123.55	0.50	102.48
MS040	/5	0.05	84.62	0.33	77.96	1.90	137.37	0.55	108.27
EP001	/PT	0.11	100.00	0.16	100.00	2.10	100.00	0.48	100.00
EP001	/2	0.04	92.68	0.28	112.58	1.32	45.82	0.27	81.10
EP001	/3	0.04	92.77	0.22	106.72	1.79	73.49	0.45	97.68
EP001	/4	0.05	93.71	0.04	88.51	1.69	66.53	0.31	84.70
EP001	/5	0.05	93.94	0.01	85.81	1.91	83.03	0.37	90.03
JW009	/PT	0.04	100.00	0.08	100.00	1.84	100.00	0.40	100.00
JW009	/2	0.02	97.73	0.01	92.87	1.35	61.14	0.17	79.61
JW009	/3	0.02	98.31	0.01	93.24	1.40	64.53	0.27	87.59
JW009	/4	0.02	98.12	0.06	97.34	1.83	98.46	0.47	107.36
JW009	/5	0.02	98.27	0.06	97.43	2.06	124.30	0.70	135.66
KB037	/PT	0.12	100.00	0.02	100.00	1.78	100.00	0.20	100.00
KB037	/2	0.04	92.45	0.19	118.65	0.96	43.91	0.23	103.25
KB037	/3	0.01	89.72	0.05	103.82	0.56	29.38	0.08	88.38
KB037	/4	0.09	97.14	0.36	140.99	1.30	61.66	0.30	110.52
KB037	/5	0.08	96.18	0.09	108.06	1.14	52.81	0.34	114.51
IT041	/PT	0.04	100.00	0.35	100.00	2.08	100.00	0.41	100.00
IT041	/2	0.02	98.46	0.94	180.04	2.07	99.25	0.50	109.97
IT041	/3	0.02	97.87	0.13	80.53	1.40	50.94	0.18	79.57
IT041	/4	0.02	98.27	0.16	82.57	1.13	38.58	0.31	90.76
IT041	/5	0.13	109.75	1.01	193.29	1.70	68.15	0.51	110.13
SK011	/PT	0.04	100.00	0.45	100.00	2.34	100.00	0.34	100.00
SK011	/2	0.05	101.61	0.03	65.31	1.94	67.57	0.40	106.88
SK011	/3	0.07	102.74	0.34	89.00	1.85	61.39	0.36	101.77
SK011	/4	0.01	97.09	0.27	83.03	2.10	78.94	0.23	89.99
SK011	/5	0.01	97.24	0.47	101.87	2.18	85.86	0.28	94.46

APPENDIX 8

PCR ELISA Data (Non-Rejectors)

Patients	Sample	IL-5	IL-5 %C	IL-13	IL-13 %C	GrB	GrB %C	FasL	FasL %C
DR013	/PT	0.07	100.00	0.02	100.00	2.08	100.00	0.26	100.00
DR013	/2	0.05	98.31	0.37	142.26	1.94	87.42	0.33	106.82
DR013	/3	0.02	95.50	0.65	188.99	2.12	104.71	0.36	111.13
DR013	/4	0.01	94.55	0.03	101.46	1.94	86.94	0.21	95.65
DR013	/5	0.01	94.46	0.59	176.65	1.78	74.56	0.34	107.90
BT016	/PT	0.06	100.00	0.06	100.00	1.32	100.00	0.28	100.00
BT016	/2	0.01	95.74	0.01	94.93	1.28	96.18	0.26	97.82
BT016	/3	0.01	95.46	0.03	97.00	1.61	134.18	0.19	91.21
BT016	/4	0.02	96.46	0.58	168.96	1.48	117.82	0.25	97.68
BT016	/5	0.00	94.79	0.07	101.11	1.87	174.28	0.27	99.05
JS017	/PT	0.50	100.00	0.33	100.00	2.27	100.00	0.54	100.00
JS017	/2	0.15	70.19	0.27	94.65	1.90	68.87	0.38	85.68
JS017	/3	0.10	66.83	0.25	92.82	1.22	34.99	0.41	88.07
JS017	/4	0.03	62.10	0.26	93.15	2.06	80.86	0.31	79.57
JS017	/5	0.02	61.45	0.25	92.13	0.93	26.28	0.19	70.65
RE022	/PT	0.04	100.00	0.00	100.00	1.05	100.00	0.18	100.00
RE022	/2	0.12	108.49	0.02	102.22	1.90	232.80	0.37	120.50
RE022	/3	0.07	102.43	0.01	100.45	0.09	38.17	0.11	93.38
RE022	/4	0.02	97.68	0.00	0.00	1.37	137.23	0.15	97.14
RE022	/5	0.06	101.46	0.22	124.86	2.22	320.91	0.72	171.77
SM026	/PT	0.04	100.00	0.13	100.00	1.99	100.00	0.37	100.00
SM026	/2	0.03	98.96	0.01	88.87	1.34	52.10	0.34	97.82
SM026	/3	0.04	99.60	0.05	92.77	0.51	22.82	0.33	96.75
SM026	/4	0.01	96.85	0.00	0.00	0.56	23.98	0.02	70.54
SM026	/5	0.01	96.66	0.01	89.40	1.22	46.49	0.25	88.78
PG007	/PT	0.10	100.00	0.12	100.00	2.15	100.00	0.48	100.00
PG007	/2	0.01	91.99	0.01	89.00	1.14	36.51	0.20	75.16
PG007	/3	0.10	100.35	0.57	156.83	1.69	63.07	0.55	106.88
PG007	/4	0.03	93.57	0.54	151.66	1.60	57.90	0.37	89.45
PG007	/5	0.16	106.88	0.65	169.81	1.76	67.91	0.60	111.91
RF020	/PT	0.04	100.00	0.36	100.00	2.39	100.00	0.54	100.00
RF020	/2	0.04	100.50	0.14	80.41	2.27	88.16	0.48	93.85
RF020	/3	0.01	97.87	0.54	119.60	2.17	79.77	0.43	89.67
RF020	/4	0.01	97.58	0.02	71.32	0.21	11.26	0.10	64.60
RF020	/5	0.01	97.43	0.05	73.79	2.29	89.72	0.16	68.45
FO003	/PT	0.06	100.00	0.71	100.00	0.40	100.00	0.26	100.00
FO003	/2	0.00	0.00	0.03	50.54	0.02	68.76	0.10	85.86
FO003	/3	0.02	96.37	0.06	52.13	0.05	70.79	0.16	90.71
FO003	/4	0.01	95.12	0.12	55.46	0.03	68.94	0.21	95.12
FO003	/5	0.00	94.46	0.41	73.68	1.87	436.01	0.55	134.72
BM024	/PT	0.07	100.00	0.05	100.00	2.24	100.00	0.21	100.00
BM024	/2	0.02	95.65	0.00	95.65	1.27	37.81	0.15	94.08
BM024	/3	0.03	96.51	0.02	96.95	1.95	74.34	0.14	93.75
BM024	/4	0.03	96.90	0.26	123.24	1.63	54.04	0.32	111.96
BM024	/5	0.03	96.66	0.20	116.18	2.11	87.77	0.49	133.31

APPENDIX 8

PCR ELISA Data (Non-Rejectors)

Patients	Sample	IL-5	IL-5 %C	IL-13	IL-13 %C	GrB	GrB %C	FasL	FasL %C
KB027	/PT	0.08	100.00	0.28	100.00	1.36	100.00	0.18	100.00
KB027	/2	0.02	94.65	0.07	81.14	2.36	271.83	0.50	136.96
KB027	/3	0.04	95.93	0.25	97.09	0.31	34.92	0.08	90.26
KB027	/4	0.04	96.03	0.40	113.15	0.49	42.17	0.06	88.87
KB027	/5	0.02	93.89	0.96	196.50	0.23	32.43	0.24	105.71
CA030	/PT	0.16	100.00	0.20	100.00	1.39	100.00	0.07	100.00
CA030	/2	0.05	89.05	0.46	130.28	1.64	128.85	0.13	106.18
CA030	/3	0.11	94.60	0.60	149.56	1.76	144.77	0.27	121.41
CA030	/4	0.05	89.27	0.63	154.88	1.92	170.92	0.47	149.33
CA030	/5	0.01	85.56	0.19	99.55	2.18	221.11	0.36	133.24
PC018	/PT	0.03	100.00	0.13	100.00	2.28	100.00	0.64	100.00
PC018	/2	0.01	98.02	0.06	92.59	1.68	55.27	0.33	72.98
PC018	/3	0.02	99.00	0.11	97.68	2.29	101.92	0.42	79.81
PC018	/4	0.04	100.30	0.23	109.58	2.23	95.12	0.40	78.19
PC018	/5	0.01	97.82	0.00	0.00	0.48	16.68	0.11	58.63
RW031	/PT	0.02	100.00	0.25	100.00	1.71	100.00	0.15	100.00
RW031	/2	0.01	98.66	0.04	80.98	1.87	117.41	0.22	106.72
RW031	/3	0.02	99.25	0.00	0.00	0.90	44.18	0.03	89.00
RW031	/4	0.03	100.80	0.18	93.19	1.58	87.46	0.28	113.54
RW031	/5	0.16	115.08	0.43	119.96	1.79	108.38	0.32	118.47
GK032	/PT	0.09	100.00	0.34	100.00	2.20	100.00	0.29	100.00
GK032	/2	0.04	95.08	0.12	80.57	0.68	21.90	0.20	91.67
GK032	/3	0.10	101.01	0.52	120.50	2.23	103.05	0.38	109.97
GK032	/4	0.09	99.30	0.25	91.53	1.54	51.79	0.44	116.82
GK032	/5	0.05	95.89	0.68	141.48	1.72	61.75	0.53	127.44
JR039	/PT	0.03	100.00	0.29	100.00	1.32	100.00	0.45	100.00
JR039	/2	0.04	101.16	0.06	79.25	2.21	243.63	0.41	95.74
JR039	/3	0.03	99.90	0.06	79.37	0.54	46.07	0.14	73.34
JR039	/4	0.05	102.28	0.16	87.90	0.34	37.68	0.21	78.94
JR039	/5	0.02	99.25	0.19	90.03	1.89	177.54	0.48	102.84
CB042	/PT	0.05	100.00	0.23	100.00	1.09	100.00	0.23	100.00
CB042	/2	0.05	100.75	0.17	93.94	2.14	284.91	0.36	113.37
CB042	/3	0.04	99.75	0.21	97.73	0.87	80.09	0.18	95.22
CB042	/4	0.04	99.00	0.21	98.12	0.99	90.17	0.31	107.84
CB042	/5	0.01	96.80	0.04	83.15	0.87	79.97	0.04	82.53
DM044	/PT	0.50	100.00	0.47	100.00	2.22	100.00	0.56	100.00
DM044	/2	0.37	87.68	0.21	77.22	2.17	95.22	0.51	94.98
DM044	/3	0.28	80.61	0.19	75.20	1.73	61.63	0.37	82.37
DM044	/4	0.04	62.94	0.19	75.65	1.62	55.13	0.21	70.15
DM044	/5	0.05	64.02	0.58	112.02	2.11	89.40	0.46	90.21
RM047	/PT	0.09	100.00	0.27	100.00	2.39	100.00	0.75	100.00
RM047	/2	0.15	105.81	0.10	84.32	2.25	87.42	1.00	127.19
RM047	/3	0.02	93.33	0.00	0.00	0.09	10.11	0.07	50.33
RM047	/4	0.08	99.20	0.04	79.14	2.40	101.41	0.55	81.26
RM047	/5	0.13	104.29	0.18	91.16	2.02	69.56	0.95	122.02

APPENDIX 9

PCR ELISA Data (Rejectors)

Patients	Sample	IL-2	IL-2 %C	IL-4	IL-4 %C	IFN-G	IFN-G %C	IL-10	IL-10 %C
NR034	/PT	0.06	100.00	1.73	100.00	1.09	100.00	0.03	100.00
NR034	/2	0.05	99.25	1.73	100.15	1.85	214.04	0.25	125.73
NR034	/1R1	0.01	94.93	2.24	165.78	0.72	69.11	0.49	158.49
NR034	/1R2	0.01	95.74	2.34	184.41	1.81	204.62	0.33	136.00
NR034	/1R3	0.03	97.29	2.11	146.52	1.25	117.47	0.12	109.58
EK043	/PT	1.37	100.00	2.27	100.00	2.21	100.00	1.78	100.00
EK043	/2	0.16	29.57	1.26	36.48	1.82	67.30	1.50	75.73
EK043	/1R1	0.16	29.78	0.35	14.73	2.12	91.35	2.07	133.24
EK043	/1R2	0.04	26.34	0.55	17.85	0.18	13.12	0.09	18.48
EK043	/1R3	0.05	26.49	1.26	36.39	0.37	15.79	0.05	17.76
JH029	/PT	0.08	100.00	1.36	100.00	1.89	100.00	0.21	100.00
JH029	/2	0.01	93.89	0.55	44.42	1.33	56.78	0.60	147.62
JH029	/3	0.07	99.35	1.13	79.49	1.17	48.41	0.48	130.93
JH029	/4	0.59	167.03	1.71	141.62	2.04	115.95	1.62	413.09
JH029	/1R1	0.03	95.79	1.24	88.51	1.34	57.55	1.50	363.28
JH029	/1R1a	0.01	93.75	1.06	74.56	0.63	28.29	0.19	98.81
JH029	/1R2	0.04	96.03	1.41	105.50	2.00	111.85	1.39	327.23
JH029	/1R3	0.05	97.09	1.53	119.07	2.10	122.88	1.04	231.06
JM008	/PT	0.02	100.00	1.31	100.00	1.62	100.00	0.03	100.00
JM008	/2	0.03	100.55	0.00	27.21	1.28	71.46	0.63	182.67
JM008	/3	0.02	99.60	0.02	27.76	1.62	99.80	0.15	112.75
JM008	/4	0.01	98.17	1.76	157.78	2.00	146.96	0.21	119.18
JM008	/1R1	0.10	108.11	0.06	28.77	1.78	117.64	0.73	200.77
JM008	/1R2	0.00	97.78	1.83	169.55	2.22	182.76	1.25	340.25
VH025	/PT	1.58	100.00	1.69	100.00	2.32	100.00	1.01	100.00
VH025	/2	0.53	35.03	1.02	51.15	1.97	70.75	1.89	241.33
VH025	/3	0.63	38.56	0.94	47.57	2.24	93.05	0.96	95.17
VH025	/1R1	0.73	42.66	0.09	20.26	1.89	65.51	1.84	227.73
VH025	/1R2	1.35	79.41	1.80	111.74	2.32	100.65	2.01	271.69
VH025	/1R3	1.21	68.76	1.27	66.10	2.15	85.04	1.76	210.33
AB021	/PT	1.17	100.00	1.85	100.00	2.21	100.00	1.70	100.00
AB021	/2	0.61	56.78	1.71	86.85	2.23	102.38	1.23	62.31
AB021	/3	0.54	53.34	1.89	103.72	2.08	87.99	1.93	126.24
AB021	/4	0.41	46.84	1.61	78.35	1.86	71.14	0.79	40.27
AB021	/1PR	0.37	44.78	2.08	126.36	1.96	78.15	0.44	28.42
AB021	/1R1	0.17	36.73	1.11	47.50	2.25	104.08	1.39	73.71
AB021	/1R2	1.03	87.02	2.31	158.41	2.25	104.86	1.36	71.39
AB021	/1R3	1.02	86.11	1.86	101.16	2.21	100.30	0.93	46.56
MP015	/PT	0.47	100.00	1.85	100.00	2.09	100.00	1.50	100.00
MP015	/2	0.47	99.60	1.21	52.65	1.68	66.46	2.22	206.27
MP015	/3	0.18	74.30	1.26	55.63	1.28	44.53	1.77	130.67
MP015	/4	0.26	80.29	1.50	70.79	1.29	45.00	1.90	149.48
MP015	/1R1	0.24	78.70	0.20	19.21	1.34	47.21	1.86	144.20
MP015	/1R1a	0.03	63.95	1.66	82.78	0.42	18.85	2.23	207.82

APPENDIX 9

PCR ELISA Data (Rejectors)

Patients	Sample	IL-2	IL-2 % C	IL-4	IL-4 % C	IFN-G	IFN-G % C	IL-10	IL-10 % C
NH023	/PT	0.53	100.00	0.95	100.00	2.05	100.00	1.02	100.00
NH023	/2	0.07	63.48	0.18	46.16	1.85	81.79	0.94	91.71
NH023	/3	0.55	102.48	0.72	79.77	1.77	75.88	0.69	71.39
NH023	/1PR	0.22	73.68	0.86	91.44	1.66	67.67	0.63	67.54
NH023	/1R1	0.21	73.12	0.09	42.17	1.84	81.06	1.22	122.26
NH023	/2R1	0.36	84.20	0.09	42.21	1.86	82.74	1.26	126.68
NH023	/2R2	0.13	67.17	1.18	125.29	1.95	90.71	1.41	147.77
NH023	/2R3	0.07	63.29	0.86	91.30	2.06	101.21	0.30	48.48
BR006	/PT	0.61	100.00	1.05	100.00	1.99	100.00	0.65	100.00
BR006	/2	0.66	105.65	0.10	38.67	1.27	48.65	1.75	300.12
BR006	/3	0.46	86.37	0.86	82.53	1.70	74.56	0.80	116.59
BR006	/1R1	0.12	61.45	0.13	39.87	1.30	50.18	1.63	265.91
BR006	/1R2	1.12	166.53	2.11	289.50	2.34	142.19	1.99	383.44
BR006	/2R1	0.52	91.85	0.95	90.21	2.26	130.80	1.83	327.23
BR006	/2R2	0.83	124.92	1.47	152.50	2.09	109.91	1.70	286.91
PH005	/PT	0.82	100.00	1.09	100.00	1.56	100.00	1.18	100.00
PH005	/2	0.45	69.49	0.39	49.34	0.73	43.47	2.12	256.38
PH005	/3	0.48	71.61	1.05	95.70	1.19	69.25	1.70	167.87
PH005	/4	0.27	57.78	0.78	73.09	0.64	39.93	1.09	91.76
PH005	/5	0.66	85.30	1.32	125.48	1.46	90.94	1.84	192.42
PH005	/1R1	0.37	63.67	0.02	34.23	1.58	102.33	2.04	235.26
PH005	/1R2	0.87	105.18	0.74	70.26	1.27	74.64	0.95	79.06
EJ010	/PT	1.00	100.00	2.11	100.00	1.09	100.00	0.61	100.00
EJ010	/2	1.05	104.66	0.65	23.28	1.13	104.55	0.94	139.17
EJ010	/3	0.74	76.57	1.32	45.32	0.20	41.02	0.67	106.45
EJ010	/4	1.54	170.83	2.02	91.35	0.95	87.33	2.10	442.38
EJ010	/1PR	1.20	122.14	2.36	128.40	1.55	159.04	1.45	230.48
EJ010	/1R1a	0.48	59.34	1.61	61.12	1.01	92.26	1.89	360.33
TG014	/PT	0.77	100.00	1.01	100.00	1.05	100.00	1.37	100.00
TG014	/2	0.48	74.49	0.80	81.42	0.92	87.94	2.01	189.93
TG014	/3	0.18	55.71	0.79	80.29	0.13	39.83	1.74	145.21
TG014	/1R1	0.13	52.97	0.62	67.88	0.82	79.25	2.09	205.65
TG014	/1R2	0.32	63.83	1.70	199.57	1.48	154.50	1.73	143.05
FB045	/PT	1.03	100.00	1.84	100.00	1.39	100.00	0.30	100.00
FB045	/2	1.01	98.17	1.21	53.21	1.17	80.01	0.49	120.80
FB045	/3	0.07	38.14	1.99	116.77	0.48	40.27	0.21	91.03
FB045	/1R1	0.05	37.44	2.14	135.12	0.49	40.68	1.74	421.65
FB045	/1R2	0.01	35.93	1.17	51.20	0.03	25.54	0.02	75.09
FB045	/1R3	0.44	55.32	2.07	126.30	2.29	245.71	0.50	122.32
EC019	/PT	0.26	100.00	0.95	100.00	1.36	100.00	1.51	100.00
EC019	/2	0.24	98.46	1.06	110.96	1.09	75.96	1.99	162.26
EC019	/3	0.07	82.49	1.03	108.17	0.90	63.29	1.95	155.35
EC019	/1R1	0.19	92.91	0.01	39.14	1.06	73.90	2.28	215.65
EC019	/1R2	1.00	210.22	0.61	71.28	1.39	102.79	0.85	51.61
EC019	/1R3	0.98	205.44	0.89	93.61	1.70	139.79	0.89	53.50

APPENDIX 9

PCR ELISA Data (Rejectors)

Patients	Sample	IL-2	IL-2 % C	IL-4	IL-4 % C	IFN-G	IFN-G % C	IL-10	IL-10 % C
ER046	/PT	1.05	100.00	1.87	100.00	2.08	100.00	0.80	100.00
ER046	/2	0.56	61.51	1.64	79.25	2.03	94.84	1.18	146.30
ER046	/3	0.31	47.85	1.52	70.08	1.12	38.35	0.82	101.51
ER046	/1R1	0.55	60.47	1.85	97.68	1.27	44.64	1.99	326.91
ER046	/2R1	0.76	74.83	1.73	86.94	2.11	103.10	1.47	195.62
ER046	/2R2	0.58	62.53	1.39	61.91	1.69	67.54	0.82	101.56
ER046	/2R3	0.23	44.00	1.51	69.98	1.77	73.16	0.98	119.30

APPENDIX 9

PCR ELISA Data (Rejectors)

Patients	Sample	IL-5	IL-5 %C	IL-13	IL-13 %C	GrB	GrB %C	FasL	FasL %C
AB021	/PT	0.04	100.00	0.43	100.00	2.20	100.00	0.28	100.00
AB021	/2	0.03	98.81	0.76	139.79	1.69	59.84	0.36	108.33
AB021	/3	0.04	100.60	0.58	116.42	1.83	69.28	0.11	83.69
AB021	/4	0.01	96.80	0.99	175.15	1.44	46.91	0.04	78.51
AB021	/1PR	0.02	97.97	1.14	204.42	1.56	52.83	0.09	81.95
AB021	/1R1	0.01	97.63	0.16	76.84	1.17	35.90	0.24	95.89
AB021	/1R2	0.02	98.71	1.29	238.10	2.08	89.09	0.31	103.05
AB021	/1R3	0.01	97.58	0.74	136.34	1.53	51.35	0.32	103.20
EK043	/PT	0.17	100.00	0.93	100.00	2.29	100.00	0.34	100.00
EK043	/2	0.04	87.77	0.54	67.33	1.67	53.55	0.25	91.76
EK043	/1R1	0.03	87.02	0.02	40.13	1.41	41.46	0.23	89.76
EK043	/1R2	0.03	86.59	0.04	41.09	0.02	10.24	0.04	74.56
EK043	/1R3	0.02	85.98	0.04	41.05	0.13	11.51	0.06	76.11
JM008	/PT	0.03	100.00	0.72	100.00	2.09	100.00	0.75	100.00
JM008	/2	0.05	101.66	-0.01	0.00	0.72	25.45	0.41	71.28
JM008	/3	0.13	110.24	0.04	50.81	2.23	115.08	0.70	95.60
JM008	/4	0.27	127.00	1.29	177.71	2.17	108.17	0.64	89.49
JM008	/1R1	0.02	98.76	0.04	50.61	2.28	120.68	0.76	100.80
JM008	/1R2	0.12	109.47	1.10	145.64	2.27	120.32	0.64	90.21
MP015	/PT	0.04	100.00	0.33	100.00	2.08	100.00	0.71	100.00
MP015	/2	0.00	0.00	0.32	98.86	0.46	19.74	0.75	103.98
MP015	/3	0.00	96.46	0.75	152.27	0.52	21.07	0.78	106.66
MP015	/4	-0.01	0.00	0.77	155.50	1.10	37.64	0.40	72.72
MP015	/1R1	0.02	97.92	0.19	86.68	1.46	53.74	0.85	114.40
MP015	/1R2	0.01	96.90	0.44	111.68	0.11	13.99	0.16	57.55
TG014	/PT	0.06	100.00	0.12	100.00	1.95	100.00	0.44	100.00
TG014	/2	0.05	99.30	0.07	95.36	1.69	77.61	0.24	82.28
TG014	/3	0.01	95.17	0.07	94.70	1.41	58.57	0.09	70.86
TG014	/1R1	0.02	96.46	0.14	101.66	1.17	45.98	0.17	76.80
TG014	/1R2	0.01	94.65	0.54	151.66	1.95	100.20	0.35	91.53
PH005	/PT	0.02	100.00	0.06	100.00	1.29	100.00	0.22	100.00
PH005	/2	0.02	99.80	0.01	95.17	0.90	67.74	0.26	104.13
PH005	/3	0.02	100.00	0.13	107.09	0.93	69.77	0.16	94.46
PH005	/4	0.03	100.50	0.08	102.07	0.39	40.66	0.06	85.38
PH005	/5	0.03	100.55	0.45	148.36	1.88	180.13	0.39	118.71
PH005	/1R1	0.01	99.05	0.00	94.70	1.34	105.02	0.20	98.31
PH005	/1R2	0.01	98.86	0.08	102.38	1.72	154.03	0.12	90.44
BR006	/PT	0.19	100.00	0.08	100.00	1.45	100.00	0.29	100.00
BR006	/2	0.01	83.53	0.01	93.47	0.87	56.10	0.14	86.07
BR006	/3	0.02	84.16	0.08	99.95	1.49	104.24	0.18	89.54
BR006	/1R1	0.02	83.90	0.01	92.77	1.06	68.32	0.04	78.08
BR006	/1R2	0.03	84.92	0.52	155.35	0.90	58.22	0.22	93.71
BR006	/2R1	0.04	85.68	0.18	110.30	1.02	65.41	0.25	96.51
BR006	/2R2	0.02	83.78	0.42	140.07	0.83	54.12	0.16	87.90

APPENDIX 9

PCR ELISA Data (Rejectors)

Patients	Sample	IL-5	IL-5 %C	IL-13	IL-13 %C	GrB	GrB %C	FasL	FasL %C
EC019	/PT	0.05	100.00	0.22	100.00	2.32	100.00	0.40	100.00
EC019	/2	0.02	100.00	0.24	100.00	1.80	100.00	0.12	100.00
EC019	/3	0.03	100.45	0.76	167.36	1.86	106.40	0.21	109.91
EC019	/1R1	0.06	103.72	0.00	0.00	1.47	72.47	0.19	107.09
EC019	/1R2	0.03	100.20	0.43	120.68	0.88	40.15	0.40	131.92
EC019	/1R3	0.07	104.39	0.39	116.42	1.00	44.91	0.16	103.98
VH025	/PT	0.06	100.00	0.26	100.00	1.96	100.00	0.37	100.00
VH025	/2	0.02	96.27	0.12	87.50	1.59	68.66	0.26	89.58
VH025	/3	0.04	97.34	0.24	98.02	1.52	64.27	0.20	83.78
VH025	/1R1	0.02	96.32	0.03	79.61	1.70	76.87	0.32	94.51
VH025	/1R2	0.10	103.46	0.90	190.12	2.00	103.87	0.60	125.48
VH025	/1R3	0.02	95.93	0.53	131.65	1.68	75.50	0.46	109.58
EJ010	/PT	0.05	100.00	0.87	100.00	2.27	100.00	0.46	100.00
EJ010	/2	0.03	97.87	0.08	45.36	1.90	68.80	0.29	85.00
EJ010	/3	0.01	95.93	0.20	51.22	1.59	50.94	0.07	68.15
EJ010	/4	0.10	104.66	0.88	101.01	1.98	74.60	0.40	94.41
EJ010	/1PR	0.84	218.80	1.26	147.92	2.24	97.34	0.45	99.25
EJ010	/1R1a	0.02	96.61	0.31	57.15	2.34	107.04	0.34	89.14
FB045	/PT	0.08	100.00	0.68	100.00	0.61	100.00	0.24	100.00
FB045	/2	0.10	101.82	0.37	73.42	0.05	57.04	0.11	87.42
FB045	/3	0.04	96.08	0.90	123.99	0.05	57.21	0.19	94.70
FB045	/1R1	0.05	96.56	1.41	206.89	0.51	90.57	0.10	86.76
FB045	/1R2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
FB045	/1R3	0.00	0.00	0.67	98.56	2.23	503.80	0.66	152.20
NR034	/PT	0.01	100.00	0.19	100.00	0.38	100.00	0.06	100.00
NR034	/2	0.07	106.40	0.33	114.57	0.18	82.08	0.18	112.47
NR034	/1R1	0.00	0.00	0.94	210.75	0.07	73.86	0.12	106.08
NR034	/1R2	0.00	99.05	0.66	158.96	1.76	397.49	0.24	119.42
NR034	/1R3	0.00	0.00	0.68	163.39	0.94	174.80	0.16	111.02
ER046	/PT	0.27	100.00	0.65	100.00	2.19	100.00	0.48	100.00
ER046	/2	0.14	87.77	0.98	138.40	2.12	93.05	0.49	100.80
ER046	/3	0.08	82.32	1.02	144.92	1.67	59.48	0.19	74.83
ER046	/1R1	0.13	86.94	1.35	200.17	2.10	91.53	0.36	88.60
ER046	/2R1	0.03	78.58	0.70	104.97	2.25	106.29	0.24	78.35
ER046	/2R2	0.05	79.57	0.47	83.40	2.06	87.63	0.30	83.53
ER046	/2R3	0.06	81.14	0.68	102.43	2.16	97.09	0.33	86.07
JH029	/PT	0.15	100.00	0.62	100.00	0.62	100.00	0.36	100.00
JH029	/2	0.01	87.24	0.05	56.72	0.16	63.32	0.28	92.54
JH029	/3	0.05	90.48	0.56	94.08	0.09	58.92	0.21	85.98
JH029	/4	0.26	111.46	1.06	155.89	2.16	465.53	0.28	92.68
JH029	/1R1	0.26	111.74	0.95	139.79	2.22	493.82	0.29	93.43
JH029	/1R1a	0.01	87.55	0.06	57.15	1.74	305.72	0.08	75.77
JH029	/1R2	0.00	86.50	0.58	96.27	2.32	549.04	0.65	134.11
JH029	/1R3	0.11	96.18	0.80	120.38	2.31	541.41	0.72	142.69

APPENDIX 9

PCR ELISA Data (Rejectors)

Patients	Sample	IL-5	IL-5 %C	IL-13	IL-13 %C	GrB	GrB %C	FasL	FasL %C
NH023	/PT	0.02	88.16	0.20	65.67	2.08	428.45	0.25	89.76
NH023	/2	0.02	88.03	0.01	54.47	1.60	266.58	0.30	93.99
NH023	/3	0.02	88.25	0.19	65.02	1.26	189.74	0.28	92.64
NH023	/1PR	0.09	94.60	0.41	81.06	1.57	259.09	0.37	100.50
NH023	/1R1	0.02	88.43	0.02	55.02	1.88	351.31	0.48	113.03
NH023	/2R1	0.04	89.45	0.02	54.91	1.94	375.47	0.52	117.70
NH023	/2R2	0.03	88.56	0.86	127.12	2.26	517.07	0.65	134.18
RE022	/2R3	0.01	87.46	0.01	54.17	2.24	505.56	0.21	86.20

APPENDIX 10

Cytokine Ratios (Non-Rejectors)

Patients	Sample	IL-4/IL-2	IL-10/IL-2	IFN-G/IL-2	IL-4/IFN-G	IL-10/IFN-G	IL-10/IL-4
PC018	/PT	1.62	0.62	2.39	0.68	0.26	0.38
PC018	/2	2.34	1.08	4.31	0.54	0.25	0.46
PC018	/3	3.92	2.73	6.15	0.64	0.44	0.70
PC018	/4	4.04	2.58	5.65	0.71	0.46	0.64
PC018	/5	0.78	0.04	2.04	0.38	0.02	0.05
JS017	/PT	1.07	0.83	1.15	0.93	0.72	0.78
JS017	/2	1.86	2.30	3.24	0.58	0.71	1.23
JS017	/3	2.77	2.47	3.25	0.85	0.76	0.89
JS017	/4	6.03	3.97	4.59	1.31	0.86	0.66
JS017	/5	4.98	4.29	7.61	0.65	0.56	0.86
CA030	/PT	22.94	0.99	21.03	1.09	0.05	0.04
CA030	/2	8.81	1.49	10.13	0.87	0.15	0.17
CA030	/3	3.81	2.00	4.65	0.82	0.43	0.52
CA030	/4	21.63	15.07	25.65	0.84	0.59	0.70
CA030	/5	17.94	5.51	24.29	0.74	0.23	0.31
KB027	/PT	15.66	0.96	23.82	0.66	0.04	0.06
KB027	/2	6.81	9.51	21.54	0.32	0.44	1.40
KB027	/3	6.57	1.23	13.03	0.50	0.09	0.19
KB027	/4	8.01	0.73	11.63	0.69	0.06	0.09
KB027	/5	6.92	0.39	6.20	1.12	0.06	0.06
SM026	/PT	2.18	0.12	3.60	0.60	0.03	0.05
SM026	/2	0.40	1.68	4.89	0.08	0.34	4.14
SM026	/3	1.18	0.46	2.53	0.47	0.18	0.39
SM026	/4	3.53	0.08	5.65	0.62	0.01	0.02
SM026	/5	6.62	1.05	9.96	0.66	0.11	0.16
RE022	/PT	16.92	1.98	42.45	0.40	0.05	0.12
RE022	/2	0.00	0.57	1.13	0.00	0.51	252.57
RE022	/3	1.87	2.99	22.39	0.08	0.13	1.60
RE022	/4	1.12	7.81	10.18	0.11	0.77	6.99
RE022	/5	2.44	3.20	2.74	0.89	1.17	1.31
BM024	/PT	5.59	2.78	13.52	0.41	0.21	0.50
BM024	/2	0.35	0.47	2.69	0.13	0.18	1.36
BM024	/3	5.56	5.17	7.20	0.77	0.72	0.93
BM024	/4	4.13	0.32	5.04	0.82	0.06	0.08
BM024	/5	1.31	1.25	1.78	0.74	0.70	0.95
RF020	/PT	2.27	2.01	1.99	1.14	1.01	0.88
RF020	/2	2.65	4.36	3.95	0.67	1.10	1.64
RF020	/3	4.01	3.25	2.76	1.45	1.18	0.81
RF020	/4	5.01	3.63	4.98	1.01	0.73	0.72
RF020	/5	7.18	1.09	3.43	2.10	0.32	0.15
BT016	/PT	1.41	0.70	1.87	0.75	0.38	0.50
BT016	/2	0.35	6.29	7.26	0.05	0.87	17.76
BT016	/3	2.77	5.25	6.20	0.45	0.85	1.90
BT016	/4	3.72	3.51	3.82	0.97	0.92	0.94
BT016	/5	4.36	4.19	5.08	0.86	0.83	0.96

APPENDIX 10

Cytokine Ratios (Non-Rejectors)

Patients	Sample	IL-4/IL-2	IL-10/IL-2	IFN-G/IL-2	IL-4/IFN-G	IL-10/IFN-G	IL-10/IL-4
EP001	/PT	3.24	2.21	3.40	0.95	0.65	0.68
EP001	/2	5.54	4.21	6.13	0.90	0.69	0.76
EP001	/3	3.36	3.56	3.15	1.07	1.13	1.06
EP001	/4	1.85	0.33	2.63	0.70	0.13	0.18
EP001	/5	1.13	2.43	2.66	0.43	0.91	2.15
JW009	/PT	2.01	3.65	1.86	1.08	1.96	1.81
JW009	/2	0.54	1.57	2.68	0.20	0.58	2.93
JW009	/3	0.52	2.99	2.87	0.18	1.04	5.70
JW009	/4	1.84	3.57	2.64	0.70	1.35	1.94
JW009	/5	2.04	4.37	4.06	0.50	1.08	2.14
SK011	/PT	2.47	2.13	2.02	1.22	1.05	0.86
SK011	/2	0.41	1.86	3.11	0.13	0.60	4.57
SK011	/3	1.64	2.30	2.43	0.67	0.95	1.41
SK011	/4	2.64	3.13	2.87	0.92	1.09	1.19
SK011	/5	10.93	20.40	9.52	1.15	2.14	1.87
DR013	/PT	6.64	2.88	10.97	0.61	0.26	0.43
DR013	/2	7.97	16.26	12.30	0.65	1.32	2.04
DR013	/3	17.87	28.61	18.08	0.99	1.58	1.60
DR013	/4	12.26	9.75	19.86	0.62	0.49	0.80
DR013	/5	5.41	7.86	5.44	1.00	1.45	1.45
PG007	/PT	3.05	1.49	3.58	0.85	0.42	0.49
PG007	/2	0.46	5.57	2.75	0.17	2.03	12.11
PG007	/3	2.24	2.23	1.73	1.30	1.29	1.00
PG007	/4	2.36	0.78	2.64	0.89	0.30	0.33
PG007	/5	2.28	2.17	2.14	1.06	1.01	0.95
FO003	/PT	8.94	11.87	6.74	1.32	1.76	1.33
FO003	/2	0.14	18.17	4.52	0.03	4.02	131.00
FO003	/3	0.09	13.75	3.87	0.02	3.55	148.19
FO003	/4	2.40	9.67	5.16	0.47	1.87	4.02
FO003	/5	4.65	9.28	3.69	1.26	2.51	2.00
RW031	/PT	6.07	0.92	11.44	0.53	0.08	0.15
RW031	/2	1.13	5.59	9.83	0.11	0.57	4.95
RW031	/3	4.66	2.06	25.18	0.19	0.08	0.44
RW031	/4	2.62	4.09	7.43	0.35	0.55	1.56
RW031	/5	13.06	9.11	16.53	0.79	0.55	0.70
GK032	/PT	4.79	0.39	5.78	0.83	0.07	0.08
GK032	/2	3.34	2.21	5.33	0.63	0.41	0.66
GK032	/3	7.00	4.03	9.52	0.73	0.42	0.58
GK032	/4	61.59	51.37	74.14	0.83	0.69	0.83
GK032	/5	1.43	0.60	1.87	0.76	0.32	0.42
KB037	/PT	23.25	10.21	21.13	1.10	0.48	0.44
KB037	/2	20.93	13.39	24.39	0.86	0.55	0.64
KB037	/3	24.89	5.11	9.43	2.64	0.54	0.21
KB037	/4	4.01	1.66	4.64	0.86	0.36	0.42
KB037	/5	5.03	3.77	7.23	0.70	0.52	0.75

APPENDIX 10

Cytokine Ratios (Non-Rejectors)

Patients	Sample	IL-4/IL-2	IL-10/IL-2	IFN-G/IL-2	IL-4/IFN-G	IL-10/IFN-G	IL-10/IL-4
AA033	/PT	10.23	0.73	11.67	0.88	0.06	0.07
AA033	/2	3.31	3.58	10.00	0.33	0.36	1.08
AA033	/3	16.53	17.74	45.55	0.36	0.39	1.07
AA033	/4	1.10	0.36	1.43	0.77	0.25	0.33
AA033	/5	5.02	0.97	9.25	0.54	0.10	0.19
DK036	/PT	4.33	0.91	5.05	0.86	0.18	0.21
DK036	/2	15.83	4.75	16.64	0.95	0.29	0.30
DK036	/3	11.75	2.00	26.04	0.45	0.08	0.17
DK036	/4	3.23	0.99	6.70	0.48	0.15	0.31
DK036	/5	5.43	3.29	20.23	0.27	0.16	0.61
LH035	/PT	64.67	2.11	36.33	1.78	0.06	0.03
LH035	/2	55.04	85.67	83.17	0.66	1.03	1.56
LH035	/3	37.90	31.48	85.10	0.45	0.37	0.83
LH035	/4	74.48	57.06	71.60	1.04	0.80	0.77
LH035	/5	12.68	12.30	15.63	0.81	0.79	0.97
IL038	/PT	1.25	1.24	10.88	0.12	0.11	0.99
IL038	/2	2.17	136.39	251.17	0.01	0.54	62.95
IL038	/3	2.00	28.50	1363.50	0.00	0.02	14.25
IL038	/4	42.96	5.64	168.28	0.26	0.03	0.13
IL038	/5	78.05	13.28	66.03	1.18	0.20	0.17
JR039	/PT	11.69	1.54	18.14	0.64	0.09	0.13
JR039	/2	0.60	0.91	2.16	0.28	0.42	1.51
JR039	/3	11.62	1.40	19.71	0.59	0.07	0.12
JR039	/4	63.92	19.27	99.38	0.64	0.19	0.30
JR039	/5	13.17	2.90	15.26	0.86	0.19	0.22
MS040	/PT	1.45	0.18	1.69	0.86	0.11	0.12
MS040	/2	3.09	1.95	2.23	1.38	0.87	0.63
MS040	/3	3.14	1.48	4.49	0.70	0.33	0.47
MS040	/4	3.05	1.77	1.74	1.76	1.02	0.58
MS040	/5	5.60	5.05	2.46	2.27	2.05	0.90
IT041	/PT	3.61	0.33	6.46	0.56	0.05	0.09
IT041	/2	11.77	10.05	13.91	0.85	0.72	0.85
IT041	/3	12.25	1.10	36.10	0.34	0.03	0.09
IT041	/4	9.88	1.71	10.73	0.92	0.16	0.17
IT041	/5	14.72	10.95	18.22	0.81	0.60	0.74
CB042	/PT	27.13	7.49	52.28	0.52	0.14	0.28
CB042	/2	2.82	1.17	10.60	0.27	0.11	0.41
CB042	/3	17.44	11.20	14.58	1.20	0.77	0.64
CB042	/4	2.90	2.10	5.80	0.50	0.36	0.72
CB042	/5	11.28	4.67	18.00	0.63	0.26	0.41
DM044	/PT	1.06	1.28	1.80	0.59	0.71	1.21
DM044	/2	2.68	2.55	3.02	0.89	0.85	0.95
DM044	/3	2.00	1.02	2.59	0.77	0.39	0.51
DM044	/4	5.69	0.63	2.66	2.14	0.24	0.11
DM044	/5	4.80	2.41	8.63	0.56	0.28	0.50

APPENDIX 10

Cytokine Ratios (Non-Rejectors)

Patients	Sample	IL-4/IL-2	IL-10/IL-2	IFN-G/IL-2	IL-4/IFN-G	IL-10/IFN-G	IL-10/IL-4
RM047	/PT	12.60	2.73	18.76	0.67	0.15	0.22
RM047	/2	0.08	18.44	22.81	0.00	0.81	239.67
RM047	/3	0.08	2.46	18.46	0.00	0.13	29.50
RM047	/4	1.57	0.73	4.28	0.37	0.17	0.46
RM047	/5	20.61	12.79	41.31	0.50	0.31	0.62

APPENDIX 11

Cytokine Ratios (Rejectors)

Patients	Sample	IL-4/IL-2	IL-10/IL-2	IFN-G/IL-2	IL-4/IFN-G	IL-10/IFN-G	IL-10/IL-4
NR034	/PT	30.12	0.44	18.94	1.59	0.02	0.01
NR034	/2	34.67	5.09	37.00	0.94	0.14	0.15
NR034	/1R1	406.82	88.36	130.82	3.11	0.68	0.22
NR034	/1R2	167.43	23.79	128.93	1.30	0.18	0.14
NR034	/1R3	70.47	3.90	41.67	1.69	0.09	0.06
EK043	/PT	1.65	1.29	1.61	1.03	0.80	0.78
EK043	/2	8.10	9.65	11.67	0.69	0.83	1.19
EK043	/1R1	2.18	12.71	13.05	0.17	0.97	5.84
EK043	/1R2	13.64	2.24	4.50	3.03	0.50	0.16
EK043	/1R3	27.64	1.10	8.03	3.44	0.14	0.04
JH029	/PT	17.86	2.71	24.89	0.72	0.11	0.15
JH029	/2	41.96	45.81	101.96	0.41	0.45	1.09
JH029	/3	16.22	6.84	16.78	0.97	0.41	0.42
JH029	/4	2.89	2.76	3.46	0.84	0.80	0.95
JH029	/1R1	37.42	45.33	40.58	0.92	1.12	1.21
JH029	/1R1a	92.48	16.87	54.70	1.69	0.31	0.18
JH029	/1R2	39.73	39.20	56.44	0.70	0.69	0.99
JH029	/1R3	32.94	22.44	45.11	0.73	0.50	0.68
JM008	/PT	55.53	1.28	68.81	0.81	0.02	0.02
JM008	/2	0.12	21.81	44.17	0.00	0.49	180.71
JM008	/3	1.21	7.69	82.82	0.01	0.09	6.38
JM008	/4	352.20	41.10	400.40	0.88	0.10	0.12
JM008	/1R1	0.58	7.16	17.53	0.03	0.41	12.32
JM008	/1R2	1833.00	1254.50	2220.00	0.83	0.57	0.68
VH025	/PT	1.07	0.64	1.46	0.73	0.44	0.60
VH025	/2	1.90	3.55	3.69	0.52	0.96	1.87
VH025	/3	1.50	1.53	3.56	0.42	0.43	1.02
VH025	/1R1	0.12	2.51	2.59	0.05	0.97	20.52
VH025	/1R2	1.33	1.49	1.72	0.77	0.87	1.12
VH025	/1R3	1.05	1.45	1.78	0.59	0.82	1.38
AB021	/PT	1.58	1.45	1.88	0.84	0.77	0.92
AB021	/2	2.83	2.03	3.68	0.77	0.55	0.72
AB021	/3	3.48	3.56	3.83	0.91	0.93	1.02
AB021	/4	3.89	1.91	4.52	0.86	0.42	0.49
AB021	/1PR	5.67	1.20	5.33	1.06	0.22	0.21
AB021	/1R1	6.53	8.22	13.24	0.49	0.62	1.26
AB021	/1R2	2.24	1.32	2.18	1.03	0.60	0.59
AB021	/1R3	1.82	0.91	2.16	0.84	0.42	0.50
MP015	/PT	3.89	3.16	4.40	0.88	0.72	0.81
MP015	/2	2.56	4.72	3.57	0.72	1.32	1.84
MP015	/3	7.11	9.94	7.21	0.99	1.38	1.40
MP015	/4	5.89	7.45	5.06	1.16	1.47	1.26
MP015	/1R1	0.84	7.93	5.70	0.15	1.39	9.39
MP015	/1R1a	60.33	81.05	15.29	3.95	5.30	1.34

APPENDIX 11

Cytokine Ratios (Rejectors)

Patients	Sample	IL-4/IL-2	IL-10/IL-2	IFN-G/IL-2	IL4IFN	IL-10/IFN-G	IL-10/IL-4
NH023	/PT	1.80	1.94	3.88	0.46	0.50	1.08
NH023	/2	2.43	12.82	25.29	0.10	0.51	5.27
NH023	/3	1.31	1.24	3.21	0.41	0.39	0.95
NH023	/1PR	3.88	2.84	7.46	0.52	0.38	0.73
NH023	/1R1	0.41	5.70	8.57	0.05	0.67	14.06
NH023	/2R1	0.25	3.54	5.23	0.05	0.68	14.31
NH023	/2R2	9.08	10.91	15.06	0.60	0.72	1.20
NH023	/2R3	12.28	4.26	29.42	0.42	0.14	0.35
BR006	/PT	1.73	1.07	3.28	0.53	0.32	0.62
BR006	/2	0.15	2.64	1.92	0.08	1.37	17.64
BR006	/3	1.86	1.74	3.68	0.50	0.47	0.93
BR006	/1R1	1.07	13.49	10.80	0.10	1.25	12.55
BR006	/1R2	1.89	1.78	2.10	0.90	0.85	0.94
BR006	/2R1	1.81	3.51	4.32	0.42	0.81	1.94
BR006	/2R2	1.77	2.05	2.51	0.71	0.82	1.16
PH005	/PT	1.34	1.44	1.91	0.70	0.76	1.08
PH005	/2	0.85	4.67	1.60	0.53	2.92	5.47
PH005	/3	2.17	3.51	2.46	0.88	1.43	1.62
PH005	/4	2.90	4.06	2.38	1.22	1.71	1.40
PH005	/5	2.01	2.78	2.22	0.90	1.25	1.39
PH005	/1R1	0.06	5.56	4.32	0.01	1.29	90.49
PH005	/1R2	0.85	1.09	1.46	0.59	0.75	1.28
EJ010	/PT	2.10	0.61	1.09	1.93	0.56	0.29
EJ010	/2	0.62	0.90	1.08	0.57	0.83	1.45
EJ010	/3	1.79	0.92	0.27	6.63	3.40	0.51
EJ010	/4	1.31	1.36	0.62	2.11	2.20	1.04
EJ010	/1PR	1.96	1.20	1.29	1.52	0.93	0.61
EJ010	/1R1a	3.36	3.93	2.09	1.60	1.88	1.17
TG014	/PT	1.31	1.78	1.36	0.96	1.30	1.36
TG014	/2	1.69	4.23	1.94	0.87	2.18	2.50
TG014	/3	4.28	9.44	0.70	6.15	13.55	2.20
TG014	/1R1	4.64	15.59	6.09	0.76	2.56	3.36
TG014	/1R2	5.31	5.39	4.63	1.15	1.16	1.02
FB045	/PT	1.79	0.29	1.35	1.32	0.22	0.16
FB045	/2	1.19	0.49	1.15	1.03	0.42	0.41
FB045	/3	30.44	3.18	7.34	4.14	0.43	0.10
FB045	/1R1	45.52	37.05	10.45	4.36	3.55	0.81
FB045	/1R2	194.83	2.67	4.25	45.84	0.63	0.01
FB045	/1R3	4.74	1.15	5.23	0.91	0.22	0.24
EC019	/PT	3.68	5.84	5.27	0.70	1.11	1.59
EC019	/2	4.35	8.21	4.47	0.97	1.83	1.89
EC019	/3	15.62	29.56	13.70	1.14	2.16	1.89
EC019	/1R1	0.08	12.32	5.73	0.01	2.15	157.17
EC019	/1R2	0.61	0.85	1.39	0.44	0.61	1.38
EC019	/1R3	0.91	0.90	1.73	0.52	0.52	1.00

APPENDIX 11

Cytokine Ratios (Rejectors)

Patients	Sample	IL-4/IL-2	IL-10/IL-2	IFN-G/IL-2	IL4IFN	IL-10/IFN-G	IL-10/IL-4
ER046	/PT	1.79	0.77	1.98	0.90	0.39	0.43
ER046	/2	2.92	2.11	3.61	0.81	0.58	0.72
ER046	/3	4.87	2.63	3.60	1.35	0.73	0.54
ER046	/1R1	3.39	3.65	2.34	1.45	1.56	1.08
ER046	/2R1	2.28	1.94	2.78	0.82	0.70	0.85
ER046	/2R2	2.41	1.41	2.92	0.83	0.49	0.59
ER046	/2R3	6.67	4.31	7.78	0.86	0.55	0.65